UV laser multiphoton dissociation studies of H_2O, NO_2 and H_2O_2

Mary Norval.

1986

THE UNIVERSITY *of* EDINBURGH

Archives of Virology 83, 3—15 (1985)

Archives of Virology

F2

Nucleic Acid Probes in Diagnosis of Viral Diseases of Man

Brief Review

By

J. K. Kulski[1] and Mary Norval[2]

[1] Department of Microbiology, The Queen Elizabeth II Medical Centre, Nedlands, Western Australia, Australia

[2] Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh, Scotland, United Kingdom

Accepted May 22, 1984

Summary

With the recent, rapid advances in recombinant DNA technology, it has become possible to consider the use of nucleic acid probes in diagnosis of human viral diseases. Several examples are discussed which employ techniques of dot blot hybridization, sandwich hybridization and *in situ* hybridization. Typing of viral strains using restriction endonuclease digestion as an epidemiological tool is considered. Finally, the present limitations of molecular hybridization are discussed, and future developments including the production of non-radioactively labeled probes, are assessed.

Introduction

Recent advances in the area of recombinant DNA technology have led to the possibility of using nucleic acid probes in the diagnosis of viral infections in man. Integrated viral sequences may be assayed, viral nucleic acids quantitated and epidemiology carried out on a molecular level. Hybridization methods may be applied to aetiopathogenic studies particularly to viral infections where viral genome is the only remaining evidence of virus infection acquired in the distant past. In addition, techniques of *in situ* hybridization permit viral nucleic acid to be detected in specific cells of a tissue section. The specificity and speed of hybridization should allow this method to be a valuable adjunct to the conventional methods used at present, such as infection of susceptible cells and growth of virus in tissue culture, electron microscopy of specimens, examination for viral antigens by using immunofluorescence or immunoenzyme conjugates, radioimmunoassay or ELISA, and serological assays (1, 16).

Three methods of recombinant DNA technology have important applications for viral diagnosis. These are molecular cloning, nucleic acid hybridization and the use of restriction enzymes to cut DNA at specific sites (20, 34). This paper outlines some of the methods currently used to prepare the viral nucleic acid probes; then consideration is given to several methods of rapid diagnosis, viral histopathology and epidemiological studies using such reagents. Finally, the future of such a molecular approach is discussed.

Preparation of Nucleic Acid Probes

Molecular cloning procedures have been especially useful for obtaining abundant supplies of those viral nucleic acid probes which cannot be propagated in tissue culture systems, such as hepatitis B virus (HBV) and human papilloma virus (HPV). These two viruses which have genomes of less than 10 kilobases have been cloned using plasmid vectors (5, 12, 17, 30). With larger M.W. viral DNAs, a collection of recombinant plasmids, bacteriophage or cosmid clones have been made containing viral DNA fragments which collectively represent the entire genome. Some of the human viral genomes that have been cloned in this way are vaccinia (3), cytomegalovirus (CMV) (21), *Herpes simplex* virus type 1 (HSV-1) (25) and type 2 (HSV-2) (24), Epstein-Barr virus (EBV) (57) and adenovirus (50). The cloned viral DNA fragments can be further digested with restriction endonucleases and then sub-cloned into plasmid vectors. The collection of different cloned fragments, called genomic libraries, are an important source of defined and undefined DNA sequences for mapping and use as diagnostic probes. Whereas plasmids, most bacteriophages and cosmids yield double-stranded (ds) recombinant probes, single-stranded (ss) recombinant probes can be obtained using M13 phage systems (41).

DNA probes also have been obtained from RNA viruses such as rotavirus (51) and influenza A (13) by synthesizing a complementary DNA (cDNA) from the viral RNA using the enzyme "reverse transcriptase". After synthesizing the homologous strand, the cDNA can be inserted into a bacterial plasmid or phage and molecularly cloned to produce recombinant DNA probes.

Viral diagnostic probes are also prepared directly from viruses which have been propagated in eukaryotic cell-culture systems (43). However, the disadvantages of using eukaryotic cells are that the yield of virus from productive infections may be very low and viral nucleic acids may be contaminated with cellular nucleic acids during the isolation procedure. Extensive genomic drift also may occur on prolonged maintenance of virus in culture especially for RNA viruses (27).

Applications to Rapid Diagnosis

Several methods have been published recently which illustrate the potential of using nucleic acid probes in viral diagnosis. In all of these,

Table 1. *Comparison of conventional detection methods and hybridization*

Virus group	Conventional detection	Detection by hybridization and RE fragment analysis	Refs.
CMV	Growth in tissue culture (several weeks) or EM. No method of distinguishing types.	Dot blot hybridization (24 hours), 5 pg vDNA detectable, quantitative. Analysis of type by RE digestion.	1, 5, 28, 48
HSV	Growth in tissue culture (several days), EM, IF, RIA or ELISA. Types distinguished serologically or by biological properties.	Dot blot hybridization (20 hours), vDNA from less than 10^3 cells detectable.	49
		Analysis of type by RE digestion or by membrane hybridization under different conditions.	10, 26, 32
EBV	Heterophil antibody test, abnormal large lymphocytes, IF and CF.	Dot blot hybridization, 6 pg vDNA detectable (20 hours).	6
Adenovirus	Growth in tissue culture (several days), RIA or IF. Types distinguished by neutralization or CF.	Sandwich hybridization (several hours), 0.2 ng vDNA detectable. Analysis of type by RE digestion or by hybridization under different conditions.	1, 14, 39, 52, 53
HBV	Antigens and antibodies detected by RIA and ELISA.	Dot blot hybridization (24 hours).	4
		1—10 pg vDNA detectable, quantitative.	8, 44
	No cell culture system available to propogate virus.	Southern blotting and hybridization after RE digestion (48 hours). Integrated and free vDNA detectable.	
HPV	Free virus detected by EM or IF but difficult to find.	Analysis of type by RE digestion.	17, 18
	No cell culture system available to propogate virus.	Southern blotting and hybridization under different conditions.	30, 33
Rotavirus	Detection by EM, PAGE, RIA or ELISA.	Dot blot hybridization (several hours), 8 pg vRNA detectable, 10—100 × more sensitive than ELISA. Analysis of type by Northern blotting under different conditions of hybridization.	22 51

v viral; EM electron microscopy; ELISA enzyme linked immunosorbent assay; IF immunofluorescence; RIA radioimmunoassay; CF complement fixation; RE restriction endonuclease; PAGE polyacrylamide gel electrophoresis

radioactively labeled probes have been prepared and used for DNA-DNA hybridization in solution or on a solid support. A comparison of conventional and hybridization procedures for the detection of several different viral groups is summarized in Table 1.

One of the first reports on the use of hybridization to type HSV isolates was by Brautigam *et al.* (7); DNA-DNA hybridization was performed in solution with the products being analyzed by hydroxyapatite chromatography. A simpler hybridization method which can detect and type HSV DNA from less than a 1000 infected cells has since been described (49). DNA extracted from cells grown in one roller tube was alkali-denatured and immobilized on nitrocellulose membranes by dot blotting. This enabled a large number of samples to be processed simultaneously. A probe was made from ^{32}P-labeled DNA fragment of HSV-1. Low stringent washing of membranes after hybridization permitted the detection of both HSV-1 and HSV-2. Rewashing of membranes under conditions of high stringency, where only a few base mis-matches are tolerated (1), allowed discrimination between types 1 and 2 as no hybridization occurred with the latter. In this study, a *Hind*III-*Eco*RI DNA fragment was used as probe although its precise location in the HSV genome was not known (49). HSV probes for hybridization need to be selected with care as HSV-1 fragments cross-hybridize to HSV-2 with varying degrees (37) and homologies may occur between viral DNA of the herpes group and cellular DNA (and mRNAs) (38). Traditionally, HSV isolates are typed by determining differences in biological properties which are not always clear cut. Serological assays are also used and recently, monoclonal antibodies have been produced that can distinguish between HSV types 1 and 2 (35).

Other dot blot hybridizations have been carried out successfully. In 1980, Brandsma and Miller reported the screening of lymphoid cells for the presence of EBV (6). The cells were spotted on nitrocellulose filters, the DNA denatured and hybridization carried out with EBV-DNA, radiolabeled by nick translation. The sensitivity was 0.06 to 0.6 genome equivalents per cell and the method quantitative. Hybridizations are of particular importance in the study of viral infections such as EBV in which the infection is either nonproductive or virions are released in low quantity.

Flores *et al.* (22) described a dot blot technique for rotaviruses which are normally detected by electron microscopy, analysis of RNA on polyacrylamide gel electrophoresis, RIA or ELISA. The stool specimens were pre-treated with fluorocarbon and extracted with phenol before denaturation by boiling and dotting on nitrocellulose membranes. However, it was also shown that the pre-treatment procedures, apart from boiling, were not essential. Hybridization was carried out using ss rotavirus RNA labeled with ^{32}P-GTP by *in vitro* transcription or with ^{125}I by chemical iodination. Good correlation was obtained with diagnosis by other methods. There was

sufficient cross-homology between different strains of rotavirus that detection of unknown strains in the specimens was possible. Asymptomatic carriers were found. The limit of detection of 8 pg of viral RNA was 10 to 100 times more sensitive than the ELISA test. Although the dot blot was very sensitive and enabled rapid screening of a large number of samples, it lacked specificity within the rotavirus genus (22). Information on the diversity of strains can be further obtained by polyacrylamide gel electrophoresis and Northern blot hybridization as human strains differ in their RNA migration patterns (51).

Increasingly, programs are being undertaken to screen for the presence of CMV in urine or other specimens from immunosuppressed patients who are receiving bone marrow, heart and kidney transplants. By conventional methods of *in vitro* culture, clinical CMV isolates often produce recognizable cytopathic effects only after several weeks and quantitation by plaque assay is too slow to be of any benefit to a patient receiving therapy. Recently, a dot blot technique has been used to detect human CMV in urine samples that were clarified, then ultracentrifuged to deposit any virus (1, 15). The pellet was alkali-denatured, neutralized and immobilized on nitrocellulose filters before hybridization was carried out with a ^{32}P-labeled CMV DNA probe containing an *Eco*RI fragment representing 1/10 of the viral genome. This assay was rapid (being completed in one day), sensitive (detecting 5 pg viral DNA) and quantitative so that the impact of antiviral therapy on the patient could be easily measured. A similar dot blot procedure has been used to detect adenovirus types (1, 14), HSV types (49), HBV (4) and enteroviruses (29) in biological specimens.

A different hybridization system has been described to detect HBV DNA in liver and serum and to determine the presence of integrated and free viral DNA in liver cells (8). DNA was extracted from needle biopsies of liver; some was digested with the restriction endonuclease *Hind*III, and the fragments separated by electrophoresis on agarose before being transferred to a nitrocellulose membrane by the Southern blot technique (47). The hybridization was carried out using cloned HBV DNA labeled with ^{32}P by nick translation and denatured. One band (3.2 kilobase position) was found in the *Hind*III digested and undigested samples from all HBe antigen-positive patients which demonstrated unintegrated viral DNA. In addition, bands corresponding to high M.W. DNA were found in some digested samples indicating integration of HBV sequences. Serum samples were phenol extracted and assayed in a similar manner for free viral DNA. This was a more sensitive assay for serum infectivity than radioimmunoassay for HBe antigen. Thus, viral DNA replication, serum infectivity, integration, and the early stages of infection and its progression may be studied using hybridization. Also, the selection and monitoring of patients for antiviral therapy can be better assessed as it seems unlikely that drugs such as adenine arabinoside or interferon could affect integrated viral genomes. Recently,

the state of HBV DNA in various childhood liver diseases has been examined in a similar way (44).

A new test called sandwich hybridization has been described which is an adaptation of the dot blot hybridization (1, 39, 52). This has been applied to the detection of adenovirus in nasopharyngeal mucus aspirates. To test for subgroup C viruses, a fragment (29—42 per cent map position) of cloned adeno-2 was denatured and immobilized on nitrocellulose filters. It was hybridized with denatured DNA from the aspirate in the presence of the probe, ^{125}I-labeled ss adeno-2 fragment (42—45.3 per cent map position) which had been cloned in the phage M13 mp7 (39). Where the aspirate DNA was homologous to adeno-2, it annealed to both filter and probe, thus binding the probe to the filter. The sensitivity of the technique (5×10^6 molecules or 0.2 ng of adenovirus DNA) was similar to that of a radioimmunoassay for viral hexon protein (1). It is possible to manipulate the probes so that reagents may be prepared with either group or strain specificity and the technique is quantitative if suitable standards are available (1, 39, 52).

Human leukemias and lymphomas are currently being surveyed for sequences homologous to human T-cell leukemia virus DNA using probes prepared from the viral genome and provirus (56).

Applications to Viral Histopathology

Although *in situ* hybridization is not applied at the moment to rapid viral diagnosis, several examples illustrate its potential and its present use in histopathology. It is of particular value where a small proportion of cells in a population is infected or where the histological and pathological picture can be correlated with the distribution of viral genomes. In addition to localization of viral genomes, expression of viral mRNA may be monitored.

BURRELL *et al.* (11) used *in situ* hybridization to examine the replication of HBV in liver sections from patients with chronic hepatitis. ^{3}H-labeled cloned HBV DNA was used as a probe to detect HBV DNA in single cells. They were able to show focal infection suggesting cell-to-cell spread of virus, and an association between HBV replication and hepatocyte injury at the cellular level. From these results they suggested that patients showing spread of virus would be the ones most likely to benefit from treatment with antiviral agents.

Some work has been done to detect viral genomes in various neurological conditions of unknown etiology using *in situ* hybridization. SEQUIERA *et al.* (45) showed that HSV type 1 DNA was present in cells from brain smears of several patients who had died with chronic psychiatric illness and neuropathological changes. In a similar way HSV mRNA was detected in ganglion cells in sections of paravertebral sympathetic ganglia at autopsy (23). Only a small percentage of neurons within the ganglia were positive. *In situ*

hybridization has also been used to detect viral DNA or mRNA in various neoplasms such as epidermodyplasia verruciformis (36), Burkitt's lymphoma (58), nasopharyngeal carcinoma (55) and cervical carcinoma (19).

The biggest drawback at the moment to using *in situ* hybridization as a tool for rapid viral diagnosis is the time taken for the autoradiograph to develop to visualize viruses in tissue sections. This may be in the order of several days for ^{125}I and ^{32}P probes, and up to several months for ^{3}H probes. If labeling with non-radioactive reagents such as antisera to nucleic acid hybrids (40), biotin (31) or fluorochrome (2) can be further developed, then more applications for *in situ* hybridization would become apparent. A start has been made in this direction by BRIGATI *et al.* (9) who reported the use of biotin-labeled DNA or RNA probes to detect viral genomes both in tissue culture (parvovirus, polyomavirus, HSV, adenovirus, retrovirus) and in paraffin sections of tissue (HSV and adenovirus). The whole procedure was completed in less than 24 hours, although the sensitivity was not equal, as yet, to autoradiographic methods.

Applications to Viral Epidemiology

It is often important to be able to trace the source of a virus which causes an infection, to test the relatedness of different strains or to follow the genomic changes over a period of time. Restriction endonuclease digestion followed by electrophoretic separation of fragments has allowed such studies to be made in recent years. This may be allied to Southern and Northern blotting, and subsequent hybridization with nucleic acid probes.

As serological reagents which are able to distinguish between types of CMV are not available, molecular epidemiological studies have provided valuable information in this area. Restriction endonuclease digestion of CMV from random clinical isolates has indicated that no 2 viruses generate the same pattern of DNA fragments, if more than one enzyme is used. Thus, HUANG *et al.* (28) showed that endogenous virus carried by mothers during pregnancy was the most frequent source of recurrent infection, and of transmission to their babies. Re-infection with another strain occurred rarely. In much the same way SPECTOR (48) demonstrated that CMV could be spread from one baby who had viruria to another in a hospital environment. This result has obvious implications for management of patients in renal and bone marrow transplant units and in pediatric wards.

HSV isolates have also been typed (32) and classified using restriction endonuclease digestion (1) as have some adenoviruses which are not clearly identified by serological methods (53). Genome mapping has provided valuable information about the source of herpetic infection in nosocomial outbreaks (10) in temporal clusters of encephalitis cases (26) and in monitoring transmission of virus from one patient to another (46).

The analysis of diversity in strains of viruses has been illustrated by human rotaviruses isolated over a period of several years (51). The segmented viral RNAs were separated by polyacrylamide gel electrophoresis, transferred by Northern blotting to diazobenzyloxymethyl (DBM) paper and hybridized with cDNA probes under conditions of low stringency (34 per cent base mis-match tolerated) and high stringency (no more than 8 per cent base mis-match tolerated). A distinct advantage of using DBM paper instead of nitrocellulose paper is that it may be used with various probes. This method permits strains to be compared in retrospect or as new clones become available. In addition, RNA electrophoretic patterns of viral isolates can be visualized directly on gels by using ethidium bromide or a sensitive silver staining procedure (54).

A further example of the use of hybridization and restriction endonuclease analysis has been to distinguish human papilloma virus (HPV) types of which 25 are presently recognized and to associate each with a particular type of lesion (18, 33). The recent discovery of HPV-16 in cervical tumors of women using molecularly cloned viral DNA probes, HPV-6 or HPV-11, demonstrates the value of employing hybridization conditions of varying stringencies to detect and isolate new types of virus (18). Free HPV has not been found in cervical tumors using traditional techniques of viral diagnosis, such as EM, immunofluorescence or cell culture systems to propogate virus. On the other hand, many cases of non-productive viral infection and new types of virus (HPV-6, 10, 11, 16 and 18) have been identified in cervical disease using recombinant DNA procedures (42).

Present Limitations and Future Developments

This review has illustrated the significant contribution which molecular hybridization and restriction enzyme fragment analysis can make to diagnostic virology and the examples given indicate the wide ranging applications in this area, especially when allied with other techniques. Cloning and characterization of the recombinant DNAs for use as diagnostic probes involve methods which are complex and time-consuming. It is clear that their preparation will be done in research centers and molecular biology companies. The availability of commercial diagnostic kits should enable hybridization techniques to become valuable adjuncts to the more traditional diagnostic methods.

At the moment, probes are normally radio-labeled to high specific activity with γ-emitters. This procedure is expensive, produces probes of short half-life and creates difficulties in handling in a routine diagnostic laboratory. The replacement of the radioactive label by other tracer substances of at least equal sensitivity is an obvious area for development. This should enable preparations of safer and more convenient reagents which are better suited to a diagnostic laboratory. Three alternatives are currently

being tested. The first is to use antibodies against the nucleic acid hybrids which would then be detected by immunocytochemistry or ELISA (40). Secondly, biotin can be covalently attached to the C-5 of pyrimidine and incorporated into the probe *in vitro*. This may then be detected using immunofluorescence, immunoperoxidase, avidin conjugated to an enzyme or affinity chromatography, based on the specific interaction between biotin and antibiotin IgG or avidin (31). Thirdly, fluorescein and rhodamine have been coupled to the 3′-terminus of the probe giving advantages in speed and resolving power for *in situ* hybridizations (2).

It would be useful if nucleic acid probes could be generated with various specificities, such as group, type or "virulence", and these quantitated under hybridization conditions of varying stringencies. Such methods are of special interest when viruses cannot be cultured *in vitro*, when they grow very slowly, when there are no antigenic markers to type them or when numbers of samples are very large. Dots of specimen DNAs or RNAs may be prepared in countries not equipped to do hybridizations and then posted to some central laboratory where the probes are available. Pre-treatment of samples before application to nitrocellulose paper leads to some extra work but has not proved essential in some systems. A distinct advantage of dot blotting is that it allows viral sequences to be assayed either singly or as numerous samples, simultaneously. *In situ* hybridization techniques may have important applications in the field of histopathology, especially if paraffin embedded sections of tissue can be used. Integrated viral genomes can be detected which may not be expressed and therefore not demonstrable by other methods. Such studies, including the ability to characterize single cells in a tissue section, may be particularly useful in the area of tumor etiology and in disorders of unknown etiology.

Epidemiology using restriction endonuclease digestion and molecular hybridization has revealed important information for virus groups which cannot readily be typed in other ways or for which detailed genetic analysis is required. The way is now clear for antigenic relatedness, genetic mapping and pathogenesis to be compared and correlated.

Acknowledgements

J. K. Kulski is supported by the National Health and Medical Research Council (Australia). Mary Norval wishes to acknowledge the financial assistance of the Cancer Research Campaign (U.K.) and a visiting Research Fellowship of the University of Western Australia.

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Authors' address: Dr. J. K. Kulski, Department of Microbiology, The Queen Elizabeth II Medical Centre, Nedlands, Western Australia 6009.

Received December 22, 1983

Printed in Austria

Veterinary Microbiology, 10 (1984/85) 219—229
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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RUMEN PAPILLOMAS IN SHEEP

MARY NORVAL, JOANNE R. MICHIE and MARGARET V. APPS

Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG (Gt. Britain)

K.W. HEAD and R.E. ELSE

Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH (Gt. Britain)

(Accepted 4 October 1984)

ABSTRACT

Norval, M., Michie, J.R., Apps, M.V., Head, K.W. and Else, R.E., 1985. Rumen papillomas in sheep. *Vet. Microbiol.*, 10: 219—229.

Out of a sample of 200 rumens from sheep slaughtered in Edinburgh, papillomas were found in 25. They occurred as fibro-papillomas, mostly along the pillar of muscle between the dorsal and ventral rumen, and were often multiple. No inclusion bodies were seen in the lesions or papilloma virus particles on electron microscopy. Homogenisation of papillomatous tissue followed by various methods of purification did not yield identifiable virus particles, and viral DNA was not detected. Immunoperoxidase staining showed a very small number of positive cells at or on the surface of 6 out of 10 lesions examined. Thus, it seems probable that virus particles are not found in large numbers in the rumen papillomas of sheep, unlike the situation in ovine skin warts, but are present in a few epithelial cells which are rapidly shed from the surface of the mucous membrane.

INTRODUCTION

Cutaneous papillomas have been reported in a wide variety of species, including man, and are particularly common in cattle. In sheep from the British Isles they are very rare, but have been described in one report (Gibbs et al., 1975). Warts were found on the limbs of 3 sheep and the muzzle of another, and electron microscopic examination identified virus particles in the lesions with typical papilloma morphology and size. In areas of Australia with high sunlight levels, papillomas resembling verrucae vulgaris and cornified horns have been described on the unwoolled areas of the face and ears of sheep (Lloyd, 1961; Ladds and Entwistle, 1977; Vanselow et al., 1982). Virions resembling papilloma virus particles have been detected by electron microscopy after homogenisation of lesions (Vanselow et al., 1982). In addition, hyperkeratotic scales which contained similar papilloma

0378-1135/85/$03.30

virus particles have been reported on the perineum of sheep (Vanselow and Spradbrow, 1983).

There is now good evidence to link specific papilloma viruses with conversion of papillomas to carcinomas such as in the digestive tract of cattle (Jarrett et al., 1978a), eyes of cattle (Ford et al., 1982) and cervix of man (Durst et al., 1983). In sheep, cutaneous carcinomas are considered to have similar precursor lesions, with sunlight as a possible co-factor in the conversion (Vanselow et al., 1982; Vanselow and Spradbrow, 1983).

In addition to the skin, papillomas have been described in the rumen of sheep where they occur as fibropapillomas with an epithelial component and with complex branching and reuniting of rete pegs (Head, 1976). Papillomas are found in the alimentary tract only at this site, mainly in older sheep and seldom undergo malignant transformation (Head, 1976); this is unlike the situation in cattle. The ovine rumen is not often examined in Britain during routine meat inspection, so the prevalence of papillomas in this site is not known, nor is the cause. In a study of a flock of sheep from N.E. Yorkshire, McCrea and Head (1978) noted rumen papillomas in groups of up to 8 lesions, sometimes occurring with other types of tumours.

The present investigation was undertaken to ascertain the frequency of occurrence of sheep rumen papillomas, and to look for a papilloma virus in these lesions.

MATERIALS AND METHODS

Clinical material

Rumens containing 1 or several papillomas were obtained from a variety of sheep slaughtered in Edinburgh including Blackface, Halfbred and Border Leicester breeds. Normally, these rumens were selected after washing by the tripery attendant and transported to the laboratory 2—3 h later. A macroscopic examination was then made and sections of suspected papillomas retained for histological tests. The remainder was stored at - 70°C until required. In a few cases the rumens were examined within a few minutes of slaughter. Small pieces (about 2 mm^3) of any papillomas found were fixed immediately in 2.5% glutaraldehyde for subsequent ultra-structural studies using a Philips 400 TEM, while the remainder was frozen immediately in liquid nitrogen.

Preparation of homogenates and attempted purification of virus

The methods outlined by Gissmann and zur Hausen (1976) and Lancaster et al. (1976) were used. About 15 g wet weight of warts, which usually comprised 3 or 4 warts, was used. The same weight of normal tissue from a different site in the same rumens acted as a negative control. After cen-

trifugation in CsCl, the fractions with density 1.31—1.33 and 1.33—1.35 g ml^{-1} were pooled, diluted and centrifuged at 80 000 *g* for 2.5 h. The drained pellet was suspended in 1 drop distilled water and stained with uranyl acetate for examination in the electron microscope.

In addition to these methods, the procedure of Jarrett (W.F. Jarrett, personal communication, 1981) for purification of bovine papilloma virus was used. After homogenisation in Tris buffer containing 0.5% deoxycholate and 0.5% NP-40, the suspension was layered on top of a 10—20% sucrose gradient with a pad of CsCl of density 1.50 g ml^{-1} in the base, and centrifuged at 170 000 *g* for 20 min. The visible band at the interface was collected and viewed in the electron microscope after dialysis or after pelleting.

A further method used was to treat finely-minced wart tissue with guanidine thiocyanate (Chirgwin et al., 1979). Wart tissue (6 g) from 1 specimen or several pooled together, or tissue from normal rumen was homogenised by hand for a few seconds in 40 ml guanidine thiocyanate solution and dialysed immediately against a large volume of 0.01 Tris-HCl, 0.15 M NaCl, 0.1 M EDTA, pH 8.0, at room temperature. The mixture was centrifuged at 10 000 *g* for 10 min before being layered on the sucrose-CsCl gradient as above. The band at the interface was collected and centrifuged in CsCl also as above. The fractions with density 1.32—1.34 g ml^{-1} were pooled, diluted and centrifuged at 80 000 *g* for 2.5 h. The pellet was suspended in 0.1 ml distilled water and stained with uranyl acetate for examination in the electron microscope.

Extraction of viral DNA from warts

Viral DNA was extracted selectively from individual warts or pooled warts (up to 5 g wet weight) using a modified Hirt procedure (Hirt, 1967) as outlined by Orth et al. (1978), and purified by agarose gel electrophoresis. In addition, the guanidine thiocyanate procedure of Chirgwin et al. (1979) was tried. Viral DNA extracted from bovine skin warts was used as a positive control and purified SV-40 DNA (a kind gift from Dr. A. Wyllie) as a molecular weight marker.

Preparation of antisera

The method of Jensen et al. (1980) was followed using the CsCl-purified material from 5 g pooled ovine warts or normal ovine rumen to act as a negative control for each injection. New Zealand white rabbits were immunised subcutaneously 3 times at fortnightly intervals; for the first injection the material was emulsified with Freund's complete adjuvant and for the next 2 with Freund's incomplete adjuvant. The rabbits were bled 2 weeks after the final injection. Removal of cross-reacting antibodies was achieved by absorbing the sera with sheep red blood cells and was checked by immunodiffusion.

Immunoperoxidase test

Paraffin sections of ovine rumen warts and normal rumen from the same animal were prepared and the immunoperoxidase method carried out as outlined by Mukai and Rosai (1980). Endogenous peroxidase was blocked with 3% H_2O_2 in absolute methanol and non-specific background reactions were blocked with normal porcine serum at a dilution of 1/5. The rabbit antisera were used at dilutions of 1/50. The immunoperoxidase staining was done using a DAKO PAP kit followed by a counterstain with Harris's haematoxylin.

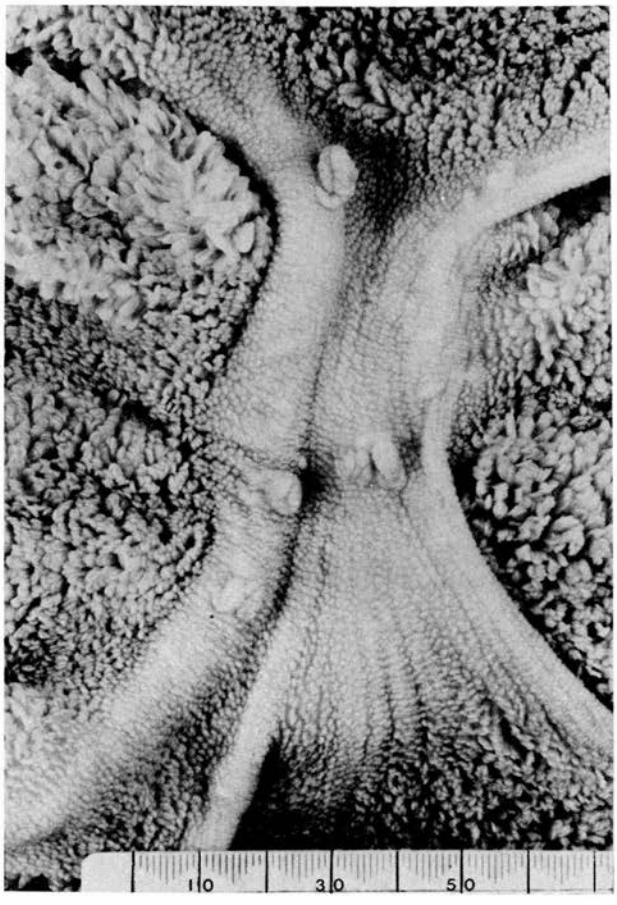

Fig. 1. Ovine papillomas distributed along the entrance of the ventral rumen (scale in mm).

RESULTS

Incidence and morphology of rumen papilloma

A survey of 200 rumens from a variety of adult ewes slaughtered in Edinburgh showed that 25 had papillomas. Due to the system of killing, it was not possible to break down these figures into distribution in individual flocks, geographical areas and breeds.

One hundred rumens containing papillomas have been examined in detail to ascertain the nature and number of lesions. Most (88%) occurred along a pillar of muscle between the dorsal and ventral rumen (Fig. 1) and the remaining 12% were found elsewhere in the rumen, especially in areas showing evidence of either current or past-healed rumenitis. Papillomas occurred singly or in multiple numbers. The average number per rumen, of those which contained papillomas, was 4.9 and the distribution is shown in Fig. 2. In 21 cases out of the 100 examined there was a prominent papilloma and several smaller ones nearby. Macroscopically the papillomas were classified as pedunculate (joined by a stalk to the mucosa of the rumen) or sessile (no stalk), and were found in the proportion of 2 : 1 approximately. They ranged in size from 2 mm to over 30 mm in diameter.

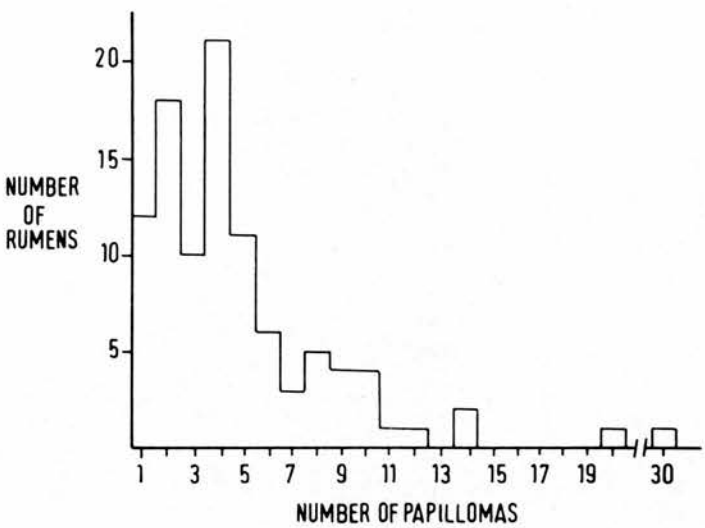

Fig. 2. The number of papillomas per rumen found in a survey of 100 papilloma-containing rumens of sheep.

Sections were cut for histological studies from a variety of 500 papillomas. In all but 4 instances, these were found to be fibro-papillomas with a branching central core of coarse collagenous connective tissue. The epithelium overlying the stroma was of similar thickness to that of the normal rumen, but showed excessive complex branching and reuniting of the rete

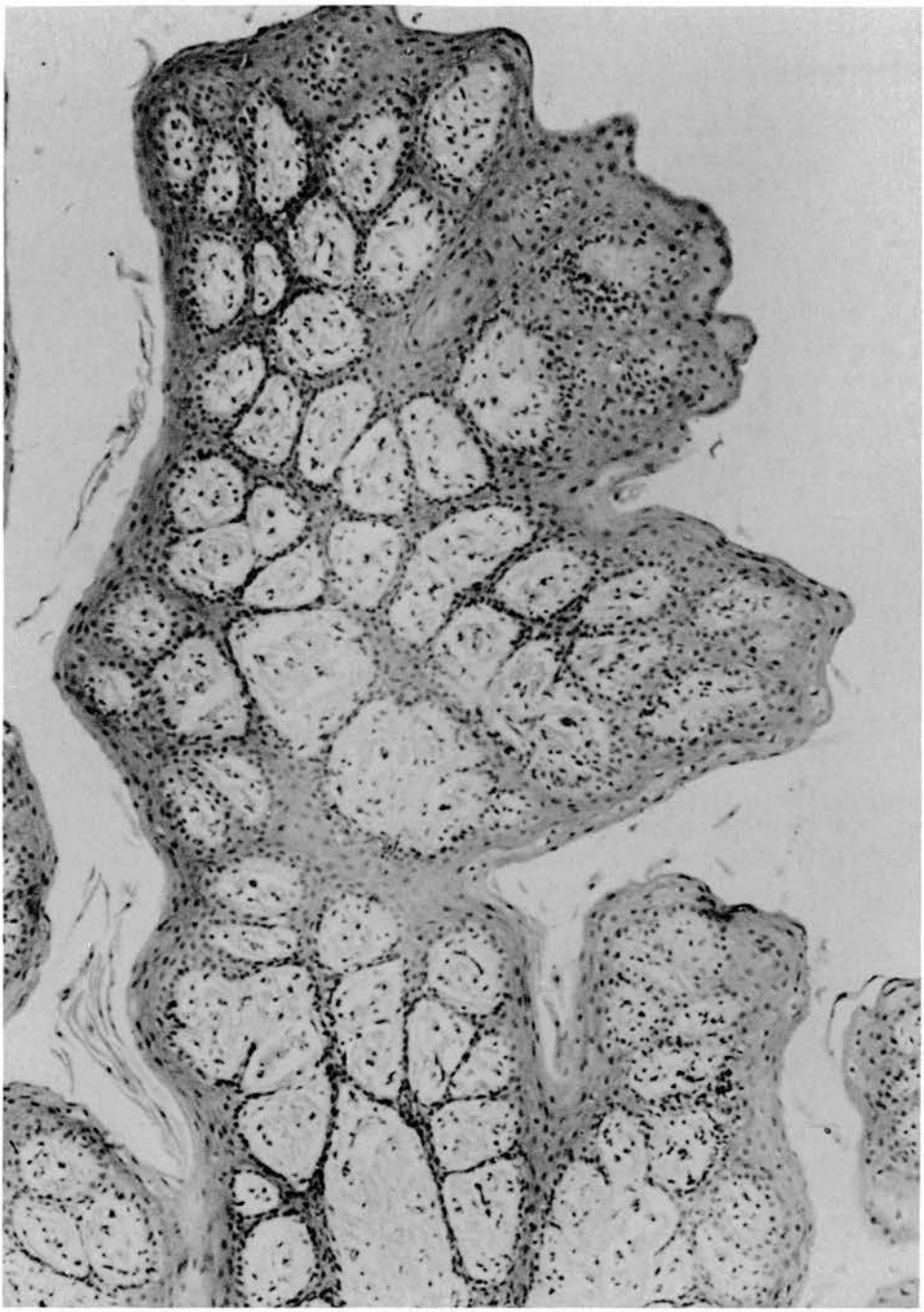

Fig. 3. Paraffin section of typical ovine rumen papilloma (haematoxylin and eosin, × 125).

pegs (Fig. 3). Epithelial cells seemed normal except for a few showing hydropic change. Keratinization was similar to the rest of the rumen as was a light and patchy infiltration of various leukocytes under the epithelium. Eosinophilic inclusion bodies were seen in over 30% papillomas examined, being present in cytoplasmic vacuoles within epithelial cells which showed hydropic change. No intranuclear inclusion bodies characteristic of papilloma virus infection were present. Normal papillae showed delicate connective tissue with large thin-walled blood and lymphatic vessels. The epithelium was thin with short stubby rete pegs. Small papillomas with diameters less than 4 mm were found to have similar structure to large, apparently normal, papillae suggesting a possible transition from these to papillomas.

In one specimen, an early squamous cell carcinoma was present in a lesion which grossly resembled a typical fibropapilloma. In 3 cases from different rumens, a squamous papilloma was present in addition to the fibropapillomas. These had very small delicate connective tissue cores which branched markedly giving long finger-like projections which were covered by stratified squamous epithelium. No mitotic figures or inclusions were seen.

Electron microscopic examination of thin sections of 30 rumen papillomas demonstrated enlarging and actively growing epithelial cells in the germinal and spinous layers with increase in tonofibrils and desmosomes. The degenerating cells in the spinous layer showed a loss of tonofibrils, detachment of desmosomes, focal necrosis, vacuoles in the cytoplasm and condensed marginated chromatin. These changes were more evident in the stratum corneum. Particles with the structure of papilloma viruses were never seen, either in crystalloid arrays or singly.

Purification of papilloma viruses and viral DNA

The papillomatous tissue from the rumen was found to be very tough, fibrous and difficult to disaggregate. Several methods were used, and a total of 20 preparations made. In addition, in case the conditions of collection and storage of the specimens were not optimal, one preparation used papillomas obtained within a few minutes of slaughter, frozen immediately in dry ice and stored at - 70°C until homogenised. Particles were never observed with the morphology of papilloma viruses in the electron microscope after negative staining with uranyl acetate.

If guanidine thiocyanate was used to disaggregate the tissue, 2 out of 5 rumen papilloma preparations were found to contain very small numbers of particles. These had a consistent diameter of about 35 nm, but were apparently without capsomeres on the surface. They were not thought to represent papilloma virus particles although no such particles were found in the other 3 specimens, or in preparations from normal rumen treated in the same way. Human papilloma virus type 1, purified on CsCl gradients

from skin scrapings of plantar warts, showed some disruption of structure on electron microscopy after guanidine thiocyanate treatment for 10 s, followed by dialysis, although the particles were still recognisable as icosahedral and had indentifiable capsomeres.

DNA has been extracted from a few human genital warts and supercoiled DNA with molecular weight typical of papillomas isolated on agarose gels (Gissmann and zur Hausen, 1980). This was tried with individual warts, ovine rumen warts of varying morphology and with pooled warts, altogether over thirty being examined. No DNA with molecular weight of papillomas was detected on agarose gels. Viral DNA extracted by the same methods from bovine skin warts gave bands of Form I, II and III DNA on agarose gels after ethidium bromide staining.

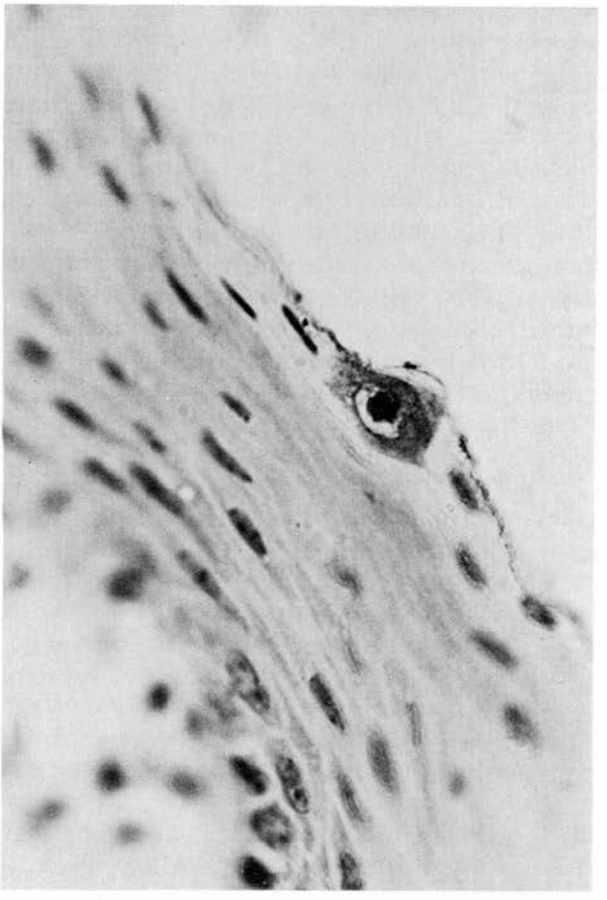

Fig. 4. Section of ovine rumen papilloma (immunoperoxidase and haematoxylin, × 675).

Immunoperoxidase staining of ovine rumen papillomas

An antiserum was prepared to the putative ovine rumen papilloma virus and used in an immunoperoxidase test on paraffin sections of rumen papillomas and normal rumen tissue. Ten specimens of warts were used, and 4 sections from each were examined. The warts varied in their pathology from sessile to pedunculate and in size from 2 to 35 mm. Six showed some reaction with the antiserum prepared against the ovine rumen papillomas and none stained with the antiserum against the normal rumen. On average, 1—3 peroxidase-stained dark brown epithelial cells were seen in each section in the keratinized upper layer on the surface of the papilloma section (Fig. 4). In addition, there were also clusters of 3—5 cells showing light brown cytoplasmic staining in the upper layers of the epithelium. Often these were found in areas where there was hydropic change. No brown cells were found in the deeper epithelial layer or in the central core of connective tissue. Papillomas which showed a positive immunoperoxidase result could not be distinguished from those giving no staining on the basis of their size, shape or histopathology. There was no staining of sections of normal rumen using either antiserum.

DISCUSSION

Only one report has indicated the presence of cutaneous warts in sheep in Britain and such lesions are known to be rare (Gibbs et al., 1975). This present survey of sheep slaughtered in Edinburgh showed the incidence of papillomas in the rumen to be 12.5%. The most common site was at the entrance of the ventral rumen where cuts and abrasions are most likely to occur. Multiple foci of papillomas were found frequently, there often being one large wart and several smaller ones. These observations may suggest an infectious aetiology for rumen papillomas with transmission by direct inoculation through minute abrasions of the surface as has been postulated for other papilloma viruses (Andrewes et al., 1978), and potential spread from an initial site of infection.

The histopathology of these warts showed them to be predominantly fibropapillomas and thus similar to the bovine oesophageal papillomas (Jarrett et al., 1978b). In cattle there is frequent conversion of multiple alimentary papillomas to carcinomas if bracken fern (*Pteridium aquilinum*), which may contain carcinogens or act as an immunosuppressive agent, is present in the diet (Jarrett et al., 1978a). The majority of sheep slaughtered in Edinburgh are grazed on bracken-infected moorlands in Scotland and the north of England but, during this work, conversion of rumen papillomas to carcinomas was found on only 1 occasion. However, it is interesting to note that the prevalence of adenocarcinoma of the small intestine in sheep slaughtered in Edinburgh has been estimated at 0.2% (J. Norval, personal communication, 1981). Also a survey of tumours occurring in

sheep grazing on the moors in North England has revealed several cases of rumen papillomas occurring in conjunction with adenocarcinoma of the small intestine (McCrea and Head, 1978).

In this study, no virus particles were detected after electron microscopy of thin sections of ovine rumen warts or after mechanical homogenisation and purification on CsCl gradients. After disruption of the tissue with guanidine hydrochloride, very small numbers of particles were seen in 2 instances which were not thought to represent papilloma viruses. In several other papillomas, such as human laryngeal warts (Lack et al., 1980) and Condylomata acuminata (Oriel and Almeida, 1970), virus particles have either not been seen at all or are present in very small numbers.

Viral DNA was not detected in any lesion. In 6 out of 10 papillomas, a few cells reacted with an antiserum raised against the putative ovine rumen papilloma virus. These cells were found on or near the surface of the epithelium, a situation similar to that found by Quick et al. (1980) and by Woodruff et al. (1980), when looking at human genital warts.

It is possible that a virus-producing cell may be shed rapidly from the surface of the mucous membrane. This would result in low quantities of viral nucleic acid and low numbers of virions being present at any one time, and would explain the results obtained here. It may be necessary in the future to resort to the sensitivity of restriction endonuclease digestion of cellular DNA followed by Southern blot techniques to detect viral DNA in ovine rumen papillomas. For this a specific ovine papilloma viral genome would be preferred as a nucleic acid probe, which could be prepared from cutaneous papillomas on sheep from Australia. This could also be used to examine the rumen papillomas for the presence of viral nucleic acid in the various layers of the epithelium and in the adenocarcinomas of the small intestine by in situ hybridization.

ACKNOWLEDGEMENT

This work was supported by the Agricultural Research Council.

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E7

UV-IRRADIATED UROCANIC ACID SUPPRESSES DELAYED TYPE HYPERSENSITIVITY TO HERPES SIMPLEX VIRUS IN MICE

James A. Ross*, PhD, Sarah E.M. Howie, PhD,
Mary Norval, PhD, Jean Maingay, BA and Thomas Simpson#, PhD
Department of Bacteriology
University of Edinburgh Medical School
Teviot Place
Edinburgh
EH8 9AG
and
#Department of Chemistry
University of Edinburgh
West Mains Road
Edinburgh
EH9 3JJ

*To whom correspondence should be addressed

This work was supported by the Medical Research Council of Great Britain.

Short title: Urocanic acid suppresses DTH to HSV-1 in mice.

Abstract

UV-radiation is known to induce a transient defect in epidermal antigen presentation which leads to the generation of antigen-specific suppression of the delayed type hypersensitivity response. The putative receptor in skin for the primary event in UV-suppression is urocanic acid which may then interact locally, or systemically, with antigen presenting cells or initiate a cascade of events resulting in suppression. We present the first direct evidence that urocanic acid, when irradiated with a dose ($96 mJcm^{-2}$) of UV-B light known to suppress the DTH response to HSV-1 in mice, can induce suppression following epidermal application or subcutaneous injection of the irradiated substance. This suppression is transferable with nylon wool-passed spleen cells.

Abbreviations used:
UV ultraviolet, CMI cell mediated immunity, LC Langerhans cell, DTH delayed type hypersensitivity, UCA urocanic acid, TNCB trinitrochlorobenzene, HSV-1 herpes simplex virus, type 1, APC antigen presenting cell, DMSO dimethylsulphoxide, UV-UCA ultraviolet-irradiated urocanic acid, NMR nuclear magnetic resonance.

Introduction

Ultraviolet (UV)-B irradiation has been shown to depress cell-mediated immunity (CMI) to tumour (1), skin sensitising (2) and viral (3) antigens encountered epidermally within a critical period after exposure. Although both the numbers and morphology of Langerhans cells (LC) in the skin are affected by UV-irradiation (4) and therapeutic UV-regimes (5), recent work has indicated that maximum suppression of the delayed type hypersensitivity (DTH) response occurs at different wavelengths from those directly affecting LC (6,7). These findings have been employed as support for the proposal of De Fabo and Noonan (8) that there is release of a photoproduct, the cis-isomer of urocanic acid (UCA), following UV irradiation of skin which may then interact with the cells of the immune system to induce suppression. Preliminary evidence from these workers (9) indicates that the DTH response to the contact sensitising agent trinitrochlorobenzene (TNCB) in mice deficient in epidermal urocanic acid cannot be suppressed by UV-B irradiation.

Previous reports from this laboratory have indicated that the DTH response to herpes simplex virus, type 1 (HSV-1) in a murine model can be suppressed by irradiation of the mice with 96 $mJcm^{-2}$ of UV-B light 3 days before sensitisation with live virus (3). The suppression of the efferent DTH response is mediated by two phenotypically distinct, $Lyl^{+}2^{-}$ and $Lyl^{-}2^{+}$, T cells (10). Epidermal APC from such mice have a reduced ability to present HSV-1 antigens to T helper cells <u>in vitro</u> (11). In this paper we investigate the possible role of urocanic acid as a factor in

mediating UV- suppression of the DTH response to HSV-1 in a murine model.

Materials and Methods

Mice - The two initial experiments were carried out using female CBA/CA mice aged 10-12 weeks obtained from the Faculty of Medicine Animal House. In all subsequent experiments C3Hf Bu/Kam male mice aged 10-12 weeks were used. The C3H mice were bred in the Department of Bacteriology animal house and all the mice maintained there.

Virus - The virus used throughout was a clinical isolate of HSV-1 whose DNA has been analysed using restriction endonucleases. The virus was cultured in Vero cells and assayed for plaque-forming units (pfu) per ml as described previously (3,10).

DTH assay - This has been described previously (3, 10). Briefly, mice were sensitised by subcutaneous injection in the right flank with 5×10^6 pfu HSV-1 grown in Vero cells. Control mice were either sensitised with an equivalent number (10^4) of sonicated, uninfected Vero cells or, in some experiments, not sensitised. The mice were tested for DTH to HSV-1 between 8 and 10 days after sensitisation. Ear thicknesses were measured before the mice were challenged by injecting 10µl (10^6 pfu) of UV-inactivated virus into each ear pinna. The ear thicknesses were again measured twenty-four hours later and the DTH calculated as the average ear increase per mouse. Suppression of DTH was determined by the formula:

$$\% \text{ suppression} = 100 - \frac{100 \times \text{net increase of experimental mice}}{\text{net increase of control mice}}$$

as described by Noonan et al (6).

Induction of suppression - To induce suppression of DTH to HSV-1, mice were irradiated for 20 minutes under two Philips TL20W/12 bulbs which gave a dose of 96mJ/cm^2 on the shaved backs of the mice as described previously (3).

Urocanic acid (Sigma (UK) Ltd.) was dissolved in a small volume of dimethylsulphoxide (DMSO) at 37°C for 5 minutes at a maximal concentration of 40 mg/ml, then diluted 10-40 fold in sterile physiological saline. This solution was poured into a large Petri dish to give a thin layer and irradiated for 20 min as for the mice. The irradiated solution was then diluted in saline as required. The UV-irradiated UCA (UV-UCA) was either painted onto the shaved, tape-stripped flanks of mice or injected subcutaneously. Four tape strips were administered using a single batch of Sellotape. The same site was used for the administration of the sensitising dose of HSV. Other mice were treated in a similar manner but using non-irradiated UCA, the vehicle or irradiated vehicle only.

Isomerisation of UCA - Nuclear magnetic resonance (NMR) spectroscopy was employed to determine the amount of UCA isomerised from the trans to cis form on irradiation as above. For this procedure the UCA was dissolved in a small volume of deuteriated DMSO at 37°C for 5 minutes then diluted in a large volume of deuterium oxide (D_2O) to a final concentration of 1 mg/ml before irradiation. The two isomers were identified from the coupling constants of the olefinic protons and their amount from the changes in the amplitude of the olefinic proton signals in the NMR. The samples were run on

a Bruker WP 80 SY nuclear magnetic resonance spectrophotometer.

Cell transfers - Cells from spleens of HSV-1 sensitised mice which had been injected with UV-UCA 5 hours before sensitisation were enriched for T cells by passage over nylon wool columns (12). The collected cells were injected intravenously into recipient mice 24 hours before ear challenge with HSV-1 as described previously (10).

Results

1. UV-induced isomerisation (trans-cis) of UCA

A time course for the isomerisation of UCA on irradiation is shown in Fig. 1. Initially there was no detectable cis-form but this was present at 24% after 20 minutes, rising to 45% at 1 hour and 74% at 4 hours. The isomer was stable on storage at room temperature, there being no reversal to the trans-form after 6 weeks. In subsequent experiments UCA irradiated for 20 minutes was used.

2. UV-irradiated urocanic acid suppresses DTH to HSV-1

This first experiment was to determine whether UV-UCA could induce suppression of the DTH response when administered 3 days or 5 hours before sensitisation with the amount normally present in the irradiated area of skin (13). Table 1 shows that UV-UCA induced marked suppression in CBA mice whether the chemical was painted onto shaved, tape-stripped skin or injected subcutaneously 5 hours or 3 days before sensitisation. Maximum suppression (57%) of the DTH response occurred when UV-UCA was injected 5 hours before a sensitising dose of HSV-1 administered at the same site. Injecting the vehicle in which the UCA was dissolved had no effect.

3. Spleen cells can transfer the UV-UCA suppression of efferent DTH to HSV-1

In order to determine the nature of the suppression induced by UV-UCA, nylon wool-passed spleen cells from UV-UCA suppressed mice (Group 5, Table 1) were transferred to syngeneic animals which had been sensitised with live virus 8 days previously.

Table 2 indicates that UV-UCA induced suppression of DTH to HSV-1 was transferable with nylon wool-passed spleen cells.

4. **Comparison of UV-UCA and non-irradiated UCA**

The effects of injecting the trans-form of UCA and of supplementing UV-irradiation of C3H mice with UV-UCA on the DTH response were examined. Table 3 indicates that there was no increase in suppression over UV-UCA alone when both UV and UV-UCA were administered together. Non-irradiated UCA had a suppressive effect on the DTH response to HSV-1 but to a lesser extent than the same quantity of UV-UCA.

5. **Calibration of the suppressive effect of UV-UCA on the DTH response to HSV-1**

To determine whether there was a relationship between the amount of UCA or UV-UCA administered and the quantity of suppression achieved, a series of dose response experiments were carried out. Representative experiments are shown in Table 4. Experiment 1 indicates that the suppression achieved by injecting UV-UCA was titratable but there was reduced suppression at the highest concentration of UV-UCA used. As before there was a lesser suppression of DTH using non-irradiated UCA compared to UV-UCA. The suppression of the DTH response generated by UV-B and 100 μg of UV-UCA was of a similar order.

Experiment 2 indicates that there was a titratable suppressive effect using small doses of UV-UCA (3 μg - 100 μg). Again, non-irradiated UCA had a suppressive effect but less than the same dose of UV-UCA. The amount of suppression achieved by 100 μg of UCA was similar to that induced by 3 μg of UV-UCA.

Nylon wool-passed spleen cells from UV-UCA treated mice from a previous experiment could transfer efferent suppression of the DTH response. The irradiated vehicle on its own had no effect on the DTH response (data not shown).

Discussion

Although UV-B irradiation is known to affect the APC function of the skin (11, 14, 15) and other organs (16) the mechanism by which the alteration in antigen handling occurs is unknown. Urocanic acid, a major component of the stratum corneum, is formed through the deamination of histidine by the enzyme histidase. Further catabolism of UCA does not occur in skin (13) as the enzyme urocanase, present in liver, is absent. UCA undergoes a UV-dependent trans to cis isomerisation (17). The action spectrum of this isomerisation shows a close fit with the action spectrum for the UV-induction of systemic suppression of contact hypersensitivity (8) and has implicated UCA in the mechanism of UV-suppression. The present paper provides the first direct evidence that the cutaneous or subcutaneous administration of the cis-isomer of urocanic acid can influence the DTH response of mice to antigens which are encountered epidermally shortly thereafter. Early evidence also indicates that this suppression is transferable with nylon wool-passed spleen cells. The evidence lends support to the hypothesis of De Fabo and Noonan (8) that the putative photoreceptor for UV-B suppression in skin is UCA. This evidence, however, does not preclude the influence of several interacting factors in the mechanism of UV-suppression.

Noonan and colleagues (6) and Morison et al (7) have elegantly demonstrated that systemic suppression of DTH to a contact sensitiser is almost certainly not generated by a UV-induced loss of LC from the UV-irradiated site or by an

alteration in LC morphology. However, it is possible that there may be a local interaction of UV-UCA with APC in the skin resulting in altered handling of antigens. This is attractive from the point of view that the soluble photoisomer, cis-UCA, must presumably be released from the cells of the stratum corneum and could interact locally with APC which would then generate a systemic suppressive response. An alternative possibility is that UV-UCA may diffuse from the epidermis to interact with the immune system at a distant site.

Our previous work indicates that a lapse of 5 hours between UV-B irradiation and sensitisation with HSV-1 is not sufficient for the initiation of a suppressed response whereas the application or injection of UV-UCA 5 hours before sensitisation is sufficient for the generation of a suppressed DTH response. We are at present addressing the question of the direct effect of UV-UCA on the functional ability of APC in vitro and in vivo.

NMR spectroscopy indicates that less than 1% of UCA as supplied is of the cis-form; this proportion, however, may be physiologically relevant although it is at the limit of resolution of the NMR technique. Also, increasing doses of non-irradiated UCA can increase the suppression induced. UV irradiation for 20 minutes significantly increases the proportion of the cis-form of UCA yet when very large doses (800 μg) of UV-UCA are administered the suppression is less than when lower doses (100 - 200 μg) are employed. Here, competitive inhibition may be important when very large amounts of trans-UCA are present.

Unlike Baden and Pathak (13) and others (17) whose early work indicated that the UV-induced isomerisation of UCA tails off as the amount of cis- and trans-isomers approaches equality our NMR spectra indicate that isomerisation under the conditions we describe continues until, at 4 hours, 74% of the UCA is of the cis- form. We propose to purify this cis- UCA and to employ it to investigate further its interaction with the cells of the immune system in the physiological range with less interference from exogenous trans-UCA. Adoptive transfer experiments are also in progress to further characterise any population of immune cells generated by UV-UCA treatment.

Acknowledgements

The authors would like to thank Dr E.C. De Fabo for his interest and encouragement.

Dr S.E.M. Howie is a Senior Research Fellow of the Medical Research Council of Great Britain.

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Fig. 1 A representative experiment showing the time course for the isomerisation (trans- cis) of urocanic acid.

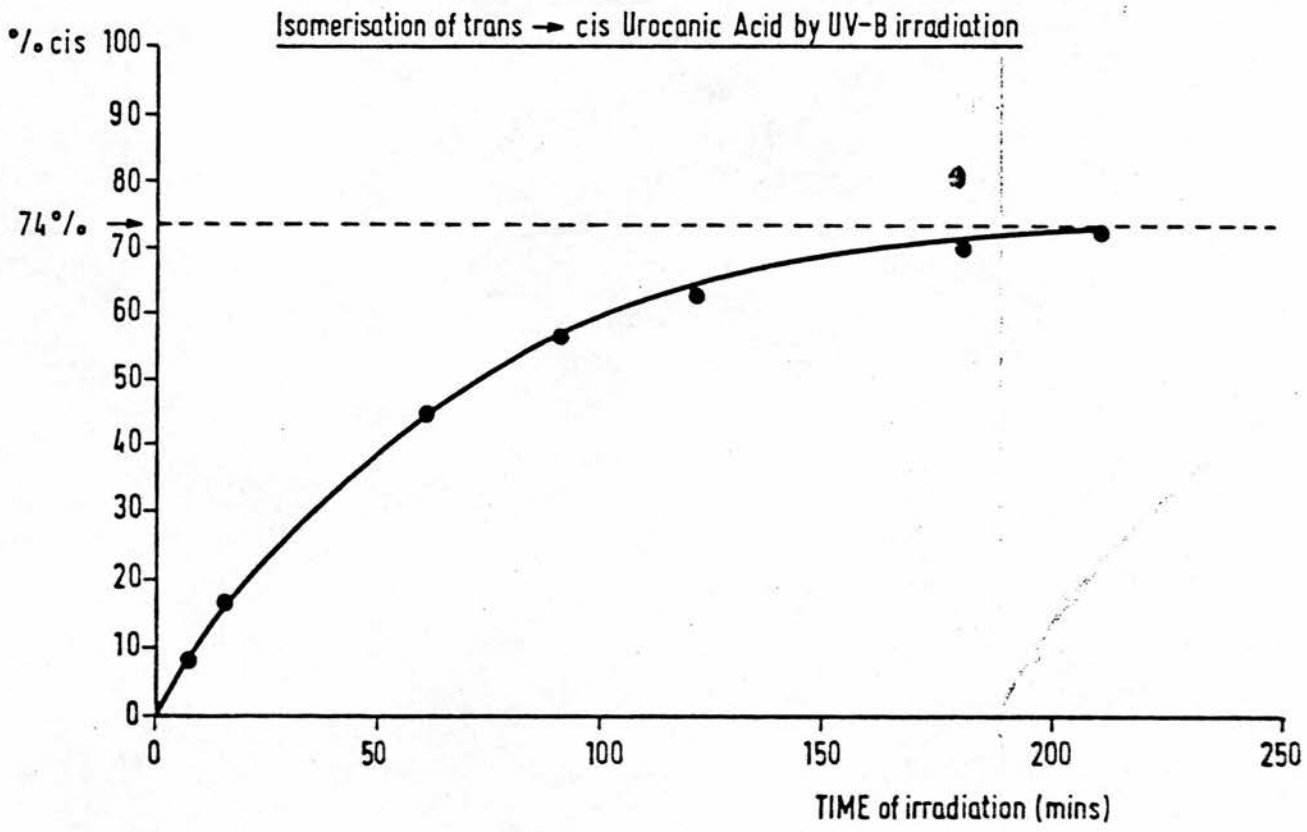

TABLE 1

UV-IRRADIATED UROCANIC ACID (UCA) SUPPRESSES DTH TO HSV-1

UV-B irradiation (3 days previously)	UV-UCA (3 days previously)	UV-UCA (5 hours previously)	sensitised with HSV at time 0 (pfu)	increase in ear thickness (mm x 10^{-2})	% suppression
NIL	NIL	NIL	NIL	1.93± 0.90 (n = 4)	-
NIL	vehicle	NIL	NIL	1.57± 0.57 (n = 3)	-
NIL	NIL	NIL	5×10^6	11.06± 0.80 (n = 4)	-
96mJcm^{-2}	NIL	NIL	5×10^6	8.50± 1.04 (n = 4)	27 ± 11
NIL	NIL	[a]200µg (injected in vehicle)	5×10^6	5.75± 0.97 (n = 4)	57 ± 10
NIL	[a]200µg (injected in vehicle)	NIL	5×10^6	7.94± 1.25 (n = 4)	33 ± 13
NIL	NIL	[b]200µg (painted in vehicle)	5×10^6	7.16± 0.65 (n = 4)	42 ± 5
NIL	[b]200µg (painted in vehicle)	NIL	5×10^6	7.08± 0.07 (n = 4)	43 ± 1

[a] = UV-UCA injected at site of subsequent sensitising dose of HSV-1.
[b] = UV-UCA applied following shaving and mild tape stripping of epidermis at site of subsequent sensitising dose of HSV.
n = number of mice per group.

TABLE 2

UV-UROCANIC ACID SUPPRESSION IS TRANSFERABLE BY SPLEEN CELLS

Sensitised with HSV at time 0 (pfu)	no. of nylon-wool passed spleen cells transferred i.v. (on Day 8)	increase in ear thickness ($mm \times 10^{-2}$)	% suppression
NIL	NIL	3.40 ± 0.34 (n = 7)	-
5×10^6	NIL	12.21 ± 0.98 (n = 7)	-
5×10^6	5×10^6	7.35 ± 0.88 (n = 5)	55 ± 9

n = number of mice per group.

TABLE 3

COMPARISON OF THE EFFECT OF UCA AND UV-UCA ON THE DTH RESPONSE TO HSV-1

UV-B irradiation (3 days previously)	UV-UCA injected (5 hours previously)	UCA injected (5 hours previously)	sensitised with HSV at time 0 (pfu)	increase in ear thickness ($mm \times 10^{-2}$)	% suppression
NIL	NIL	NIL	NIL	2.44 ± 0.54 (n = 4)	-
NIL	NIL	NIL	5×10^6	11.33 ± 1.09 (n = 3)	-
$96 mJcm^{-2}$	200ug	NIL	5×10^6	7.00 ± 0.84 (n = 5)	48 ± 9
NIL	200µg	NIL	5×10^6	6.69 ± 0.54 (n = 4)	52 ± 6
NIL	NIL	200µg	5×10^6	8.89 ± 1.68 (n = 4)	27 ± 15

n = number of mice per group.

TABLE 4

CALIBRATION OF UCA AND UV-UCA EFFECT ON DTH AND CELL TRANSFER OF SUPPRESSION

UV (3 days previously)	UV-UCA (5 hours previously)	UCA (5 hours previously)	[a]sensitised with at time 0	spleen cells transferred	Experiment 1 increase in ear thickness (mm x 10^{-2})	Experiment 1 % suppression	Experiment 2 increase in ear thickness (mm x 10^{-2})	Experiment 2 % suppression
NIL	NIL	NIL	NIL/Vero cells	NIL	2.35 ± 0.53 (n=5)	-	4.17 ± 1.00 (n=6)	-
NIL	NIL	NIL	HSV	NIL	14.38 ± 1.01 (n=6)	-	16.50 ± 0.78 (n=8)	-
96mJcm^{-2}	NIL	NIL	HSV	NIL	7.72 ± 0.70 (n=12)	55 ± 6	8.00 ± 0.72 (n=6)	65 ± 12
NIL	3 µg	NIL	HSV	NIL	N.D.		10.88 ± 0.61 (n=6)	46 ± 5
NIL	10 µg	NIL	HSV	NIL	10.20 ± 0.58 (n=6)	34 ± 5	8.70 ± 0.58 (n=6)	63 ± 5
NIL	100 µg	NIL	HSV	NIL	7.45 ± 1.54 (n=6)	60 ± 12	7.40 ± 0.53 (n=6)	74 ± 4
NIL	800 µg	NIL	HSV	NIL	9.58 ± 0.23 (n=6)	40 ± 2	N.D.	
NIL	NIL	3 µg	HSV	NIL	N.D.		12.38 ± 1.36 (n=4)	33 ± 11
NIL	NIL	10 µg	HSV	NIL	11.54 ± 0.67 (n=6)	24 ± 6	11.85 ± 1.24 (n=5)	37 ± 10
NIL	NIL	100 µg	HSV	NIL	12.00 ± 1.07 (n=6)	21 ± 9	11.50 ± 1.02 (n=4)	40 ± 8
NIL	NIL	800 µg	HSV	NIL	12.25 ± 1.16 (n=6)	25 ± 10	N.D.	
NIL	NIL	NIL	HSV	[b]2 x 10^7	N.D.		6.38 ± 1.27 (n=4)	82 ± 10

a = mice were sensitised with 5 x 10^6 pfu of HSV-1, negative controls were unsensitised or injected with 10^4 Vero cells.

b = nylon wool passed spleen cells from mice previously shown to have a suppressed DTH response to HSV-1 after 100 µg of UV-UCA transferred on day 9.

n = number of mice per group.

ND = not done

In vivo induction of suppressor T cells after presentation of HSV-1 by irradiated skin cells

S.E.M. Howie*, J.A. Ross, M. Norval and J.P. Maingay

Department of Bacteriology

Edinburgh University Medical School

Teviot Place

Edinburgh EH8 9AG.

* To whom correspondence should be addressed.

Antigens encountered by the skin induce delayed hypersensitivity (DH) reactions on subsequent exposure. DH to the epidermal virus herpes simplex type one (HSV-1) protects animals from lethal infection[1,2]. We have been studying the role of antigen presenting cells (APCs) in the generation of DH to HSV-1 and its modulation by the environmentally encountered epidermal stimulus ultraviolet-B (UV-B) irradiation. Experimentally UV-B affects APCs locally[3] and systemically[4], induces T suppressor (Ts) cells for DH[5,6] and alters HSV-1 pathogenesis by acting as a trigger for recrudescence in experimental[7] and natural[8] infections. In a murine model we reported that UV-B irradiation followed by HSV-1 infection suppresses DH to the virus <u>in vivo</u>[9] and decreases the ability of epidermal cells to present HSV-1 <u>in vitro</u>[10]. Here we report that transfer of epidermal cells from UV-B irradiated animals to naive syngeneic recipients together with live HSV-1 is sufficient to induce T cell mediated suppression of DH.

The first set of experiments (shown in Table 1) demonstrate that epidermal cells from mice irradiated 3 days previously and transferred to naive syngeneic recipients together with live HSV-1 at the same site induced suppression of DH to the virus. The same cells given simultaneously with the virus but at a different site did not cause suppression, nor did the administration of epidermal cells from non-irradiated mice. Thus it appears that external modulation of the cells involved in local antigen presentation to the immune system can determine whether DH or suppression of DH to HSV-1 is generated in otherwise untreated

animals.

Table 2 shows that the suppression generated in this way is transferable by nylon wool passed spleen cells. The suppression is of the efferent DH to HSV-1 and is specific for HSV as the response to the unrelated Semliki Forest virus (SFV) is not affected. The fine specificity of the suppression against other herpes viruses is under investigation and will be reported elsewhere.

To determine the nature of the T suppressor (Ts) cell(s), two sets of cell transfer experiments were performed. The first set utilised the monoclonal antibodies TIB104 (anti-Ly1) and TIB105 (anti-Ly2) and the results are shown in Table 3. As these antibodies are not cytotoxic, an indirect killing system was employed. Using the same system we have previously reported that the Ts cells induced by UV-B irradiation followed 3 days later by infection with HSV-1 are of two phenotypically distinct subclasses ($Ly1^+2^-$ and $Ly1^-2^+$)[11]. Interestingly, the suppressor cells induced by transfer of UV-B irradiated epidermal cells belonged only to the $Ly1^+2^-$ subclass - the more "classical" Ts cell population $Ly1^-2^+$ was absent.

To further clarify the nature of the suppressor cells a series of monoclonal antibodies directed against L3T4 (the "helper cell" determinant), Ly2 and Thy1 was employed. These antibodies are all directly cytotoxic. The results of these experiments can be seen in Table 4. The Ts population was defined as $Thy1^+$, $L3T4^+$, $Ly2^-$.

Other workers have described Ts cells for DH to HSV-1 induced by intravenous injection of either temperature sensitive mutants of

HSV-1, where Lyl^+2^- Ts cells suppressed afferent DH to HSV-1[2] or thymidine kinase deficient mutants of HSV-1 where both Lyl^+2^- and Lyl^-2^+ Ts cells suppressed afferent DH to the virus[1]. DH to HSV-1 in both systems was induced by subcutaneous infection with live unattenuated HSV-1.

Shrier et al[12] also demonstrated that different populations of Ts for DH to HSV-1 could be induced by different presentations of antigen. A temperature sensitive mutant of HSV-1 injected intravenously induced Ts cells which would only suppress afferent DH to the virus. However, intravenously injected syngeneic spleen cells infected with the same virus in vitro induced Ts cells capable of suppressing both afferent and efferent DH to HSV-1. We are investigating whether Ts cells generated in our system suppress afferent as well as efferent DH to HSV-1 and the results will be reported elsewhere.

High dose (660 mJ/cm^2) in vitro UV-B irradiation of dissociated epidermal cells has been used to reveal the presence of $I\text{-}J^+$ cells which act as APCs for the generation of Ts cells of DH to the hapten ABA[13]. However, in this system the Ts cells suppressed afferent but not efferent DH to ABA. We are currently investigating the cells in the epidermis of UV-B irradiated and normal mice using flow cytometry in an attempt to elucidate the nature of the APCs in our system.

In conclusion we have previously demonstrated that UV-B irradiation in vivo alters antigen handling by the epidermis such that Ts cells for DH to HSV-1 are generated. Two suppressor T cell

populations Lyl^+2^- and Lyl^-2^+ were demonstrated. The present report demonstrates that the ability to induce the Lyl^+2^- but not the Lyl^-2^+ population can be transferred by inoculating naive syngeneic recipients with UV-B irradiated epidermal cells at the same site and at the same time as HSV-1. The full phenotype of this Ts cell is Lyl^+2^- $L3T4^+$ $Thyl^+$. We are presently investigating the nature of UV-B induced signal which may induce the other Ts population as well as the nature of the APCs involved in the Ts generation.

Our model is unique in that the same virus was used to induce either DH or Ts for DH and represents a wild type clinical isolate of HSV-1. Similarly, in the epidermal cell transfer experiments reported here the recipient mice in which DH or Ts for DH to HSV-1 are induced are identical and otherwise untreated. The only experimental parameter which varies is the application of an external suberythemal dose of UV-B light. This irradiation is sufficient to alter the antigen handling by epidermal cells in such a way that the immune system specifically generates Ts for DH rather than DH itself. The implications of such a mechanism affecting the generation of specific immunity to epidermal pathogens particularly those which, like HSV-1, persist in the host after initial infection may be profound.

This work was funded by the Medical Research Council of which S.H. is a Senior Fellow.

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Table 1 UV-B irradiated epidermal cells inoculated at the same site as HSV-1 induces suppression of DH to the virus.

Animals sensitised with (day 0)	Increase in ear thickness (units x 10^{-2}mm) and % suppression of DH Experiment 1		Experiment 2		Experiment 3	
10^4 Vero	3.02±0.40 (n=11)		5.10±0.73 (n=5)		3.04±0.41(n=11)	
10^7 pfu HSV-1	14.23±1.00 (n=11)		12.50±0.74 (n=5)		14.56±1.23(n=4)	
UV-B 3 days previously 10^7 HSV-1 R flank	5.38±1.31[b](n= 4)	79%	ND		9.20±1.35[d](n= 4)	
Normal EC L flank[a] + 10^7 HSV-1 R flank	15.06±1.15 (n= 4)	0%	10.18±0.90[e](n=4)	31%	ND	
Normal EC R flank[a] + 10^7 HSV-1 R flank	15.13±1.30 (n= 4)	0%	10.81±1.28[e](n=4)	33%	14.70±1.47(n=6)	
UV-B EC L flank[a] + 10^7 HSV-1 R flank	14.70±1.09 (n= 5)	0%	10.35±0.82[e](n=5)	29%	ND	
UV-B EC R flank[a] + 10^7 HSV-1 R flank	7.80±0.46[c](n= 5)	57%	6.00±0.73[c](n=5)	88%	6.83±1.64[d](n=3)	

All mice challenged on Day 8. Results expressed as arithmetic mean ± 1 standard error.

n = number of experimental animals per group

a, number of epidermal cells (EC) transferred in Experiment 1 = 5 x 10^5, in Experiment 2 = 10^6, in Experiment 3 = 5 x 10^6.

b, significantly different from control group, $p < 0.01$ by t test

c, as for b, $p < 0.001$

d, as for b, $p < 0.02$

e, not significantly different from control group

LEGEND TO TABLE 1

Inbred female C3Hf Bu/Kam mice aged between 10-16 weeks, bred and maintained in the Departmental Animal House were used in all experiments.

The methods for determining DH and for separating epidermal cells have all been published elsewhere[9,10]. Briefly HSV-1 grown in Vero cells (control group) or an equivalent number of uninfected cells (background group) were injected into mice subcutaneously in the right flank. 8-10 days later ear thickness was measured and the animals challenged by injection of 10µl containing 10^6 plaque forming units (pfu) of UV inactivated HSV-1 into each ear pinna. After 24 hrs. ears were again measured and the increase in thickness calculated. To induce UV-B suppression of DH mice were irradiated with 96 $mJcm^{-2}$ UV-B (equivalent to 20 minutes exposure under two Philips TL20/12 bulbs in our system) 3 days before infection with HSV-1. For EC transfers ears were removed from normal animals or from animals which had been irradiated as above 3 days previously. The ears were split open and floated on 0.3% trypsin for 1 hr at 37°C. The trypsin was then removed by washing in phosphate buffered saline (PBS) and inactivating with 10% FCS. The epidermal sheets were separated using forceps. Single cell suspensions were made by shaking the epidermal sheets vigorously in PBS + 10% FCS in universal tubes containing glass beads. The resulting cell suspension was strained through a wire mesh. The cells were washed in PBS + 10% FCS and layered onto a discontinous 20%/50%/70% Percoll gradient. The cells at the 20%/50% interface were 80-85% viable by trypan blue exclusion and were used for the cell transfers. The cells were either mixed with HSV-1 immediately before injection into the right flank or were injected into the left flank while HSV-1 was injected into the right flank. DH was measured as above. Suppression of DH was calculated according to the formula :

$$\% \text{ suppression} = 100 - \frac{100 \times \text{net increase in experimental mice}}{\text{net increase in control mice}}$$

Table 2 The DH suppression induced by EC transfer is transferable by nylon wool passed spleen cells and is HSV specific.

Animals sensitised with (Day 0)	Animals transferred with (Day 9)	Animals challenged with (Day 10)	Increase in ear thickness (units x 10^{-2} mm) and % suppression of DH
10^4 Vero	NIL	HSV-1[a]	4.03 ± 0.63 (n = 8)
1.5 x 10^6 pfu HSV-1	NIL	HSV-1	15.82 ± 0.62 (n = 18)
UV-B 3 days previously 1.56 x 10^6 HSV-1	NIL	HSV-1	7.22 ± 0.39[b] (n = 8)
5 x 10^5 UV-B EC R flank 1.5 x 10^6 HSV-1 R flank	NIL	HSV-1	7.25 ± 0.69[b] (n = 6)
1.5 x 10^6 HSV-1	10^7 NW spleen[c] (UV-B EC + HSV-1)	HSV-1	8.59 ± 0.58[b] (n = 8)
10^4 BHK	NIL	SFV[d]	5.59 ± 0.62 (n = 8)
5 x 10^6 pfu SFV[e]	NIL	SFV	16.16 ± 0.64 (n = 14)
UV-B 3 days previously 5 x 10^6 SFV	NIL	SFV	6.09 ± 0.37[b] (n = 8)
5 x 10^5 UV-B EC R flank 5 x 10^6 SFV R flank	NIL	SFV	7.06 ± 0.54[b] (n = 8)
5 x 10^6 SFV	10^7 NW spleen[c] (UV-B EC + HSV-1)	SFV	16.31 ± 0.52 (n = 8)

a - mice challenged with 10^6 pfu UV-inactivated HSV-1 in both ear pinnae

b - significantly different from control group ($p < 0.001$ by t test)

c - mice injected with spleen cells from mice suppressed for HSV-1 DH by epidermal cell tr (cells passed through nylon wool)

d - mice challenged with 5 x 10^5 pfu UV-inactivated SFV in both ear pinnae

e - mice sensitised with 5 x 10^6 pfu UV-inactivated SFV subcutaneously in the right flank

LEGEND TO TABLE 2

DH for HSV-1 was induced and suppressed by either UV-B exposure 3 days before infection or by transfer of UV-B exposed EC at the same site and time as HSV-1 infection as described in Table 1. DH to Semliki Forest virus (SFV) was induced in an identical fashion with the exception that UV-inactivated rather than live virus was used to sensitise the mice. The SFV was passaged *in vitro* in BHK cells. Spleens were removed from mice suppressed for DH to HSV-1 by UV-B EC transfer as above 9 days after sensitisation. The spleens were homogenised and the cells passed over nylon wool columns using the method of Julius et al[14]. The cells recovered from the columns were > 95% Thy-1 positive by immunofluoresence. The nylon wool passed spleen cells were injected intravenously into mice sensitised with either HSV-1 or SFV and the mice were challenged with the appropriate virus within 24 hours. DH to SFV and HSV-1 was measured 24 hours after challenge.

Table 3 The suppression of efferent DH induced by EC transfer is transferable by nylon wool passed (NW) spleen cells of the $Ly1^{+}2^{-}$ phenotype.

Animals sensitised with (day 0)	Animals transferred with (day 9)	Increase in ear thickness (units x 10^{-2}mm) and % suppression of DH Experiment 1	Experiment 2
10^4 Vero	NIL	1.84±0.35 (n=8)	3.48±0.48 (n= 6)
3 x 10^6 pfu HSV-1	NIL	16.10±1.08 (n=8)	14.61±1.12 (n= 9)
UV-B 3 days previously 3 x 10^6 HSV-1	NIL	6.80±1.04[e] (n=8) 65%	5.23±0.45[e] (n=11) 84%
10^6 UV-B EC R flank 3x10^6 HSV-1 R flank	NIL	7.80±1.24[e] (n=8) 58%	6.33±0.69[e] (n= 6) 74%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[a] RIG + C only	8.40±1.16[e] (n=8) 54%	7.22±0.69[e] (n= 8) 66%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[b] Ly 1 (TIB 104)	14.56±1.03 (n=8) 11%	13.62±0.68 (n= 8) 9%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[c] Ly 2 (TIB 105)	8.25±0.90[e] (n= 8) 55%	7.68±0.85[e] (n= 7) 62%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[d] Ly1 + Ly2	ND	15.84±1.16 (n= 8) 0%

All mice challenged on Day 10.

n = number of experimental animals per group

a, mice injected with spleen cells from mice suppressed by epidermal-cell-transfer all passed through nylon wool and treated with anti-rat immunoglobulin and complement alone.

b, as in a, but cells also treated with anti Ly 1 monoclonal antibody TIB104

c, as in a, but cells also treated with anti Ly 2 monoclonal antibody TIB105

d, as in a, but cells also treated with both TIB104 and TIB105

e, significantly different from control group, $p < 0.001$ by t test.

LEGEND TO TABLE 3

DH and UV-B or UV-B-irradiated-epidermal-cell-transfer suppression of DH was induced and measured as in Table 1. The methods used for treating nylon wool passed spleen cells with the monoclonal antibodies TIB104 and TIB105 (from the American Type Culture Collection) have been published elsewhere[11]. Briefly, since these rat monoclonal antibodies are not directly cytotoxic an indirect killing method was used. Nylon wool passed spleen cells were incubated with the appropriate monoclonal(s) or in PBS containing 5% FCS for 1 hr on ice, the cells were then washed x 3 and incubated with anti-rat immunoglobulin antiserum for 45 minutes on ice, the cells were again washed extensively and finally incubated with complement for 30 minutes at 37°C. After the final incubation the cells were washed and the number of viable cells counted by trypan blue exclusion. Viable cells were injected intravenously into mice which had been infected with HSV-1 nine days previously.

Table 4 The transferable suppressor cell is a T cell of the $L3T4^{+}Ly2^{-}Thy1^{+}$ phenotype.

Animals sensitised with (day 0)	Animals transferred with (day 9)	Increase in ear thickness (units x 10^{-2}mm) and % suppression of DH Experiment 1	Experiment 2
10^4 Vero	NIL	4.56±0.79 (n=8)	3.33±0.17 (n=6)
3 x 10^6 pfu HSV-1	NIL	15.05±0.84 (n=10)	14.15±1.10 (n=5)
UV-B 3 days previously 3 x 10^6 HSV-1	NIL	6.95±1.13[f] (n= 5) 77%	6.54±0.75[g] (n=6) 70%
5x10^5 UV-B EC R flank 3 x 10^6 HSV-1 R flank	NIL	7.20±0.45[g] (n= 6) 75%	8.35±0.23[g] (n=5) 54%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[a] untreated	7.14±1.11[g] (n=7) 75%	7.86±0.62[g] (n=7) 58%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[b] L3T4 (YTS 191.1)	13.47±1.11[g] (n=8) 15%	13.30±1.28 (n=7) 8%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[c] Ly2 (YTS 169.4)	6.38±0.48[g] (n=8) 83%	7.96±0.49[g] (n=6) 57%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[d] L3T4 + Ly2	14.64±0.95 (n=7) 10%	14.42±0.66 (n=6) 0%
3 x 10^6 HSV-1	5 x 10^6 NW spleen[e] Thy-1 (YTS154.7)	ND	14.58±0.95 (n=6) 0%

All mice challenged on Day 10.

n = number of experimental animals per group.

a, mice injected with spleen cells from mice suppressed by epidermal-cell-transfer; cells passed through nylon wool.

b, as in a, but cells treated with anti-L3T4 monoclonal antibody YTS191.1

c, as in a, but cells treated with anti-Ly2 monoclonal antibody YTS169.4

d, as in a, but cells treated with anti-L3T4 and anti-Ly2

e, as in a, but cells treated with anti-Thy1 monoclonal antibody YTS154.7

f, significantly different from control group, $p < 0.01$ by t test

g, significantly different from control group, $p < 0.001$ by t test

LEGEND TO TABLE 4

For induction and suppression of DH and cell transfer protocols see Tables 1, 2 and 3. After passage through nylon wool columns spleen cells from epidermal-cell-transfer-suppressed mice were treated with the directly cytotoxic monoclonal antibodies YTS191.1, YTS169.4 and YTS154.7 (from SERA-LAB Ltd., Crawley Down, Sussex, U.K.) or with PBS containing 5% FCS for one hour at room temperature. The cells were then extensively washed with PBS and the number of viable cells determined by trypan blue exclusion. The cells were then injected intravenously into syngeneic mice which had been infected 9 days previously with HSV-1. After 24 hours the mice were challenged with UV-B inactivated HSV-1 as described and the 24 hour DH reaction measured.

E9

Two phenotypically distinct T cells are involved in UV-irradiated urocanic acid induced suppression of the efferent DTH response to HSV-1 *in vivo*.

James A. Ross, PhD, Sarah E.M. Howie, PhD,
Mary Norval, PhD, and Jean Maingay, BA.

Department of Bacteriology,
University Medical School,
Teviot Place,
Edinburgh, EH8 9AG.

ABSTRACT

When UV-B irradiated urocanic acid, the putative photoreceptor/mediator for UV-B suppression, is administered to mice it induces a dose dependent suppression of the delayed type hypersensitivity response to herpes simplex virus type 1 (HSV-1), of similar magnitude to that induced by UV irradiation of mice. In this study, the efferent suppression of delayed type hypersensitivity by UV-irradiated urocanic acid is demonstrated to be due to two phenotypically distinct T cells, ($Thy1^+$, $L3T4^-$, $Ly2^+$) and ($Thy1^+$, $L3T4^+$, $Ly2^-$). The suppression is specific for HSV-1. This situation parallels the generation of two distinct T suppressor cells for HSV-1 by UV irradiation of mice and provides further evidence for the involvement of urocanic acid in the generation of UV-B suppression.

Abbreviations used:

UV ultraviolet; DTH delayed type hypersensitivity; HSV-1 herpes simplex virus, type 1; APC antigen presenting cell; SFV Semliki-Forest virus; UCA urocanic acid; UV-UCA ultraviolet-irradiated urocanic acid; DMSO dimethylsulphoxide; NMR nuclear magnetic resonance; LC Langerhans cell.

INTRODUCTION

Urocanic acid (UCA), a substance present in the stratum corneum of skin, has been implicated as the photoreceptor/mediator for ultraviolet(UV)-B induced suppression of the immune response by De Fabo and Noonan (1). Urocanic acid is formed in a single step deamination of histidine by the enzyme histidase and undergoes a UV-dependent trans - cis isomerization. The photochemical properties of urocanic acid, including the ability to form photodimers and a capacity to bind to DNA in a UV-dependent manner, have recently been reviewed by Morrison (2).

We have previously reported the first direct evidence that UV-B irradiated urocanic acid (UV-UCA) induced suppression of the delayed type hypersensitivity (DTH) response to a subsequently encountered antigen, herpes simplex virus (HSV-1), in normal mice (3). This suppression was achieved by painting the skin with UV-irradiated UCA (UV-UCA), or by injecting UV-UCA subcutaneously, shortly before sensitisation with virus. Transfer of suppression to sensitised mice was performed using nylon wool-passed spleen cells from mice previously treated with UV-UCA prior to sensitisation. Preliminary evidence from Noonan, De Fabo and Morrison (4) indicates that intraperitoneal injection of cis-UCA into mice prior to sensitisation caused suppression of contact hypersensitivity on subsequent challenge.

In this paper, we investigate the nature of the suppressor cells generated by UV-UCA in a murine model of HSV infection and discuss its relevance to the mechanism of UV-B suppression.

MATERIALS AND METHODS

Mice - CH3f Bu/Kam mice aged 10-12 weeks were used throughout and each experiment comprised either all male or all female mice. The mice were bred and maintained in the Department of Bacteriology animal house.

Viruses - HSV-1. The virus used throughout was a clinical isolate of HSV-1 cultured in Vero cells and assayed for plaque forming units (pfu) per ml, as described previously (5).

Semliki Forest Virus (SFV) - Semliki Forest Virus was the generous gift of Dr A.G. Morris of the Department of Biological Sciences at Warwick University. It was maintained by *in vitro* passage in BHK cells. For the DTH experiments, SFV of known pfu per ml was irradiated with ultraviolet light for 75 minutes to inactivate the virus whilst preserving its antigenicity. This protocol induces a DTH response to SFV (unpublished results) and was used in preference to live virus inoculation.

DTH assay - This has been described previously (3,6,7). Briefly, mice were sensitised in the right flank by subcutaneous injection of a known number of pfu of HSV-1 grown in Vero cells or UV-inactivated SFV grown in BHK cells. Control mice were injected with an equivalent number of sonicated, uninfected Vero cells or UV-irradiated BHK cells. The mice were measured for DTH to HSV-1 or SFV between 8 and 14 days after sensitisation. Ear thicknesses were measured immediately before the mice were challenged by injecting 10 µl (10^6 pfu) of UV-inactivated virus into each ear pinna. The ear thicknesses were again measured 24 hours later and

the DTH calculated as the average ear increase per mouse.

Suppression of DTH was determined by the formula:

$$\% \text{ suppression} = 100 - \frac{100 \times \text{net increase of experimental mice}}{\text{net increase of control mice}}$$

as described by Noonan et al (8).

Isomerization of UCA - This has been described previously (3). Briefly, a thinly spread solution of urocanic acid (Sigma (UK) Ltd), at a concentration of 20-40 mg/ml in dimethylsulphoxide (DMSO), was irradiated under two Philips TL20W/12 UV lamps for 20 minutes giving a dose of 96 $mJcm^{-2}$ of UV in the range 270-350 nm. NMR spectroscopy previously indicated that this dose induces a 23% isomerization of UCA to the cis form (3).

Induction of suppression - This solution of UV-UCA in DMSO was then either diluted in absolute ethanol to the desired concentration and painted onto the shaved, gently tape-stripped (x4) flanks of the mice, or diluted in sterile physiological saline and injected subcutaneously. The same site was used for the administration of the sensitising dose of HSV or SFV 5 hours later. Other mice were treated in a similar manner but using the vehicle or irradiated vehicle only.

Cell transfers - Cells from the spleens of HSV-1 sensitised mice which had been painted or injected with UV-UCA 5 hours before sensitisation were enriched for T cells by passage over nylon wool columns (9). The collected cells were treated appropriately (see below) and injected intravenously into sensitised recipient mice 24 hours before ear challenge.

Cell depletions - The rat (IgG2b) monoclonal antibodies YTS 191.1 (anti-L3/T4), YTS 169.4 (anti-Lyt2) and YTS 154.7 (anti-Thy-1) (SeraLab Ltd, Crawley Down, Sussex, UK) are able to eliminate cells without adding complement *in vitro* by coating the cells and employing *in vivo* opsonisation . The nylon wool-passed spleen cells were incubated with these antibodies at a final dilution of 1:50 and at a concentration of up to 5×10^7 cells/ml for 30 minutes at room temperature. The cells were then washed twice in serum free medium, resuspended in PBS and transferred to recipient animals.

RESULTS

1. Spleen cells transfer the UV-UCA induced suppression of efferent DTH to HSV-1

The first experiment, shown in Table 1, confirms the previous finding (3) that suppression was induced by injecting 200 μg of UV-UCA (23% cis) 5 hours before sensitisation with HSV-1 at the same site. Nylon wool passed spleen cells from a parallel group of mice mediated suppression when transferred intravenously to HSV sensitised mice 24 hours before ear challenge. The efferent suppression achieved by injecting 200 μg of UV-UCA before sensitisation or transferring spleen cells from this group of mice 24 hours before challenge was comparable.

2. $(L3T4^+, Ly2^-)$ and $(L3T4^-, Ly2^+)$ T cells are responsible for the transfer of UV-UCA induced suppression of DTH to HSV-1

In order to determine the phenotype of the transferable T suppressor cells, nylon wool passed spleen cells from UV-UCA suppressed mice were treated with (a) no antibodies, (b) anti-L3/T4 monoclonal antibody, (c) anti-Ly2 monoclonal antibody, or (d) both anti-L3/T4 and anti-Ly2 antibodies simultaneously. Two representative experiments are shown in Table 2. Suppression of the DTH response was only abrogated by simultaneous treatment with both anti-Ly2 and anti-L3/T4 antibodies.

3. Specificity of the UV-UCA induced T suppressor cells DTH to HSV-1

Table 3 confirms that the suppression is due to transferable T cells of two distinct phenotypes. Suppression was only abrogated by anti-Thy-1 treatment or by a combination of anti-Ly2 and anti-L3/T4 antibody treatment. Transferring spleen cells from UV-UCA treated mice subsequently sensitised with HSV-1 5 hours later had no effect on the DTH response to an unrelated virus (SFV). The suppressor cells were therefore specific for HSV. Table 3 also indicates that UV-UCA can induce suppression of the DTH response to other viral antigens (SFV). Painting on the irradiated vehicle had no effect on the DTH response to HSV or SFV (unpublished data).

DISCUSSION

Our recent work suggests that a temporary alteration in epidermal antigen presentation due to ultraviolet light may lead to the ineffective, inadequate or inappropriate processing and presentation of viral antigens (6,7,10). This may have long term consequences for the host, particularly in the case of persistent viral infections such as herpes simplex. Irradiation with UV in the range 250-320 nm (UV-B) has been shown to influence cell-mediated immunity to tumour (11), skin sensitising (12) and viral antigens (3,6,7,10) in such a way that there is preferential generation of T cell mediated suppression for the particular antigen. The influence of UV on the generation of antigen specific responses occurs in a critical period following exposure (6). Recent work by Noonan et al (8) and Morison et al (13) indicates that significant suppression of the DTH response occurs at different wavelengths from those directly affecting the morphology and distribution of Langerhans cells (LC). These findings provide evidence that UV-immunosuppression is not due solely to a direct effect on antigen presenting cells (APC) and further implicates the photoreceptor, urocanic acid, in the mechanism of UV-B suppression (1).

Recently we have used UV-UCA (23% cis form) painted on the skin or injected subcutaneously and shown that suppression of the DTH response to the virus occurs. This UV-UCA induced suppression is dose related and of a similar magnitude to that obtained by UV-irradiation of the mice (3). In another system, Noonan and

colleagues (4) have preliminary evidence that intra-peritoneal injection of UV-UCA induces a dose-related suppression of contact hypersensitivity to 2,4,6-trinitrochlorobenzene in mice, again similar to that observed after UV-irradiation of mice.

We have now shown that two suppressor cells ($L3T4^+$, $Ly2^-$) and ($L3T4^-$, $Ly2^+$) are involved in urocanic acid induced suppression of the DTH response to HSV. The suppression is specific for HSV as transferring these suppressor cells has no effect on the DTH response to an unrelated virus (SFV). This parallels our previous finding using a suberythemal dose (96 $mJcm^{-2}$) of UV-B where two UV-induced T suppressor cells (7) ($Ly1^+$, $Ly2^-$ and $Ly1^-$, $Ly2^+$), one of which has subsequently been shown to be $L3T4^+$, $Ly2^-$ (14), are involved in the efferent suppression of DTH to HSV-1. Thus the T suppressor cell subsets generated by UV-B (96 $mJcm^{-2}$) and by UV-UCA (96 $mJcm^{-2}$) are of similar phenotype, providing further evidence that urocanic acid may be the photoreceptor/mediator for UV-B suppression.

Current work (14) indicates that only one of the UV-induced suppressor cell phenotypes ($Thy\ 1^+$, $Ly1^+$, $L3T4^+$, $Ly2^-$) is generated via the epidermal route and is presumably associated with a unique antigen presentation event occurring locally in the skin at the site of infection following UV-irradiation. The hypothesis that the other suppressor cell phenotype is generated systemically is being investigated using both UV irradiation and UV-UCA protocols. This presentation event may be associated with APC, possibly in the spleen or lymph nodes under the influence of the

cis isomer of UCA. Both spleen and lymph node APC function is known to be affected by UV-irradiation *in vivo* (15).

Urocanic acid and the immunomodulatory substance histamine are both formed in one step reactions from histidine and are structurally closely related. The use of synthetic histamine agonists (16) has indicated that histamine may activate both suppressor and contrasuppressor T cell subsets: H_2 receptor agonists tend to activate suppressor cells while H_1 receptor agonists activate contrasuppressor cells. We have previously reported that urocanic acid promotes a biphasic response (3), where high concentrations of UV-UCA induce less suppression than more physiological doses, similar to that with histamine which is dependent on concentration. It is possible that urocanic acid may fit similar receptors or utilise histamine receptors, both in the epidermis and systemically, to exert its effect, or exert part of its effect, on the immune response. This is currently being investigated as is the intriguing hypothesis that UCA in either its *cis* or *trans* form may interfere with the binding of histamine to its receptor in skin and thus may further affect the types of response generated to epidermal antigens and pathogens.

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TABLE 1 UV-UCA suppression is transferable by nylon wool passed spleen cells

UV-UCA injected (on Day 0)	Sensitised with (on Day 0, 5 hours later)	No. of nylon wool passed spleen cells transferred i.v. (on Day 8)	Increase in ear thickness ($mm \times 10^{-2}$)	% suppression
NIL	10^4 Vero cells	NIL	4.56 ± 0.79 (n=8)	-
NIL	1.5×10^6 pfu HSV	NIL	15.05 ± 0.84 (n=10)	-
200 µg	1.5×10^6 pfu HSV	NIL	8.09 ± 0.55 (n=8)	66*
NIL	1.5×10^6 pfu HSV	[a] 6×10^6	7.88 ± 0.49 (n=8)	68*

n = number of mice per group.
a = nylon wool passed spleen cells from a parallel group of mice which had received 200 µg of UV-UCA 5 hours before sensitisation and were sacrificed on Day 8.
* = significantly different from positive response by student's t test ($p<0.001$).

TABLE 2 $L3T4^+$ $Ly2^-$ and $L3T4^-$ $Ly2^+$ cells are responsible for the transfer of UV-UCA induced suppression of DTH to HSV-1.

UV-UCA painted (on Day 0)	Sensitisation (on Day 0, 5 hours later)	Nylon wool passed Spleen cells transferred (on Day 8)	Experiment 1 increase in ear thickness (mmx10^{-2})	Experiment 1 % suppression	Experiment 2 increase in ear thickness (mmx10^{-2})	Experiment 2 % suppression
NIL	10^4 Vero cells	NIL	3.32 ± 0.50(n=7)	-	4.07 ± 0.90(n=7)	-
NIL	$1.5x10^6$ HSV	NIL	12.11 ± 0.70(n=7)	-	16.95 ± 0.86(n=5)	-
200 µg	$1.5x10^6$ HSV	NIL	3.21 ± 1.33(n=7)	100*	ND	ND
NIL	$1.5x10^6$ HSV	UNTREATED[a]	5.31 ± 1.24(n=4)	77***	5.15 ± 0.93(n=5)	92*
NIL	$1.5x10^6$ HSV	anti-Ly2[b]	10.04 ± 0.54(n=7)	24**	9.50 ± 1.17(n=4)	58**
NIL	$1.5x10^6$ HSV	anti-L3/T4[c]	8.67 ± 0.81(n=7)	39**	11.50 ± 1.37(n=4)	42****
NIL	$1.5x10^6$ HSV	anti-Ly2[d] + anti-L3/T4	11.00 ± 0.48(n=6)	13[NS]	16.45 ± 1.29(n=5)	4[NS]

ND = not done.
a = No. of cells transferred in Expt 1 = 6 x 10^6 and in Expt 2 = 9 x 10^6 per mouse.
b = Spleen cells as in (a) but also treated with YTS 169.4
c = Spleen cells as in (a) but also treated with YTS 191.1.
d = Spleen cells as in (d) but also treated with YTS 169.4 and YTS 191.1
n = No. of mice per group.
Significantly different from positive response by student's t test:
* = $p<0.001$; ** = $p<0.01$; *** = $p<0.02$; **** = $p<0.05$.
NS = Not significant.

TABLE 3 Specificity and phenotype of Ts for UV-UCA suppression of DTH

UV-UCA painted on (Day 0)	Animals sensitised with (Day 0, 5 hours later)	Spleen cells transferred (Day 9)	[a]Increase in ear thickness ($mm \times 10^{-2}$)	% suppression
-	10^4 Vero	-	4.03 ± 0.63 (n=8)	-
-	10^4 BHK	-	5.59 ± 0.62 (n=8)	-
-	1.5×10^6 HSV-1	-	15.82 ± 0.62 (n=18)	-
-	5×10^6 SFV	-	16.16 ± 0.64 (n=14)	-
200 µg	1.5×10^6 HSV-1	-	8.42 ± 0.62 (n=6)	63*
200 µg	5×10^6 SFV	-	8.13 ± 0.72 (n=8)	76*
-	5×10^6 SFV	8×10^6 NW spleen[b] untreated	15.03 ± 0.80 (n=8)	11[NS]
-	1.5×10^6 HSV-1	8×10^6 NW spleen[b] untreated	7.84 ± 0.70 (n=8)	68*
-	1.5×10^6 HSV-1	8×10^6 NW spleen[c] anti-Ly2 (YTS 169-4)	8.72 ± 0.75 (n=8)	60*
-	1.5×10^6 HSV-1	8×10^6 NW spleen[d] anti-L3T4 (YTS 191-1)	8.18 ± 0.79 (n=7)	65*
-	1.5×10^6 HSV-1	8×10^6 NW spleen[e] anti-Ly2 + anti-L3T4	15.03 ± 0.67 (n=8)	7[NS]
-	1.5×10^6 HSV-1	8×10^6 NW spleen[f] anti-Thy1 (YTS 154.7)	15.75 ± 0.81 (n=8)	1[NS]

a = all mice challenged on Day 10.
b = nylon wool passed spleen cells from mice treated with 200 μg UV-UCA epidermally 5 hours before sensitisation with HSV-1 and sacrificed on Day 8.
c = as in b, but cells treated with anti-Ly2 antibody YTS 169.4.
d = as in b, but cells treated with anti-L3T4 antibody YTS 191.1.
e = as in b, but cells treated with anti-Ly2 and anti-L3T4.
f = as in b, but cells treated with anti-Thy1 antibody YTS 154.7.
n = number of experimental animals per group.
* = significantly different from appropriate positive control, $p<0.001$ by t test.
NS = not significantly different from appropriate positive control by t test.

Hypothesis

ON THE NATURE OF THE DEEP CELLULAR DISTURBANCES IN HUMAN-PAPILLOMA-VIRUS INFECTION OF THE SQUAMOUS CERVICAL EPITHELIUM

STEWART FLETCHER MARY NORVAL

Departments of Pathology and Bacteriology, University of Edinburgh

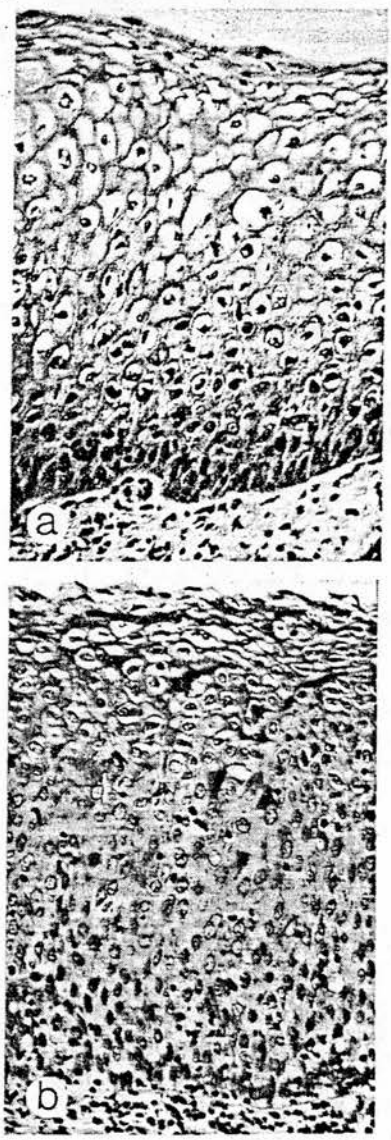
Summary In the squamous epithelium of the cervix, disturbed deep cells, usually regarded as dysplastic, often underlie superficial koilocytes. Recognition of koilocytosis as a cytopathic effect of human papilloma virus (HPV) prompts reconsideration of the state of the deep cells, which could be non-dysplastic or dysplastic. In the dysplastic states HPV could be non-causative or causative. For dysplastic causation by HPV, a cell-differentiation hypothesis reconciles the coexistence in the epithelium of a permissive HPV infection of the superficial layers (koilocytosis) and a non-permissive cellular infection of the deep layers, which may undergo transformation to dysplasia.

INTRODUCTION

IN general, cells may respond to virus infections in several

ways.[1] They may be resistant and show no change or evidence of infection. In a productive infection, however, a cytopathic effect usually develops during the replication of many complete virions which, in the case of naked viruses like human papilloma virus (HPV), are released during cell autolysis.[2] In the cervix, the superficial koilocytes are thought to die from the cytopathic effects of a productive (permissive) papilloma-virus infection: they cannot, therefore, be progenitors of a malignant cell line.

In most non-productive (non-permissive) infections, the virus enters and infects the cell. Viral reproduction stops at the stage of viral nucleic-acid replication, and virus remains as "incomplete" naked genome which ranges numerically from part of 1 genome to, perhaps, 200 genomes. With oncogenic viruses about 1 in 10^5 of infected non-permissive cells undergo transformation: in vitro their morphology becomes distinctive and they can grow in suspension and form foci over the monolayer of untransformed cells. In vivo they may be tumorigenic in susceptible animals.[3]



Superficial koilocytes, a presumptive sign of an HPV productive infection.

a, basal cells with minimal disturbance. b, deep cells with disturbances indistinguishable from moderate dysplasia. (Haemalum and eosin, reduced by a third from ×250.)

In transformation the viral genome may be integrated into the host-cell genome[4] or remain non-integrated, as free covalently closed circles. Integrated and non-integrated states may coexist. In either state the viral genome, or part of it, can be transmitted to the daughter cells on division without release of virus, thus perpetuating the transformation and any of its cellular effects. Some of the viral genome may be transcribed and translated, and "early" viral proteins may be expressed on the cellular or nuclear membranes and serve as markers of cellular transformation or neoplasia.

The non-permissive state of the transformed cells prevents replication of complete virus and concomitant cell death: transformed cells are thus enabled to generate malignant cell lines.

A mixed, semipermissive, type of infection is also recognised in cell culture. Here some cells are non-permissive, or transformed, whereas others are permissive and undergo a productive infection causing, where appropriate, cell death. Probably differences in the cells rather than differences in the virus determine the type of infection.

The in-vitro patterns of permissive and non-permissive viral replication and of transformation suggest hypotheses about the nature of the cellular changes in the squamous epithelium of the cervix in HPV infections.

SQUAMOUS-EPITHELIAL-CELL CHANGES IN PAPILLOMA-VIRUS INFECTION

In the squamous epithelium of the cervix productive HPV infection is commonplace in the form of koilocytes in the upper strata.[5-7] They may or may not be associated with disturbances of the deep cells indistinguishable microscopically from dysplasia (see figure).

Before the viral cause of koilocytic change was established[5,8-11] the nature of the disturbed cells would not have been questioned and they would have been regarded as one of the grades of dysplasia or as carcinoma-in-situ. In our present imperfect state of knowledge, that very practical view is still necessary to ensure that the patient is adequately treated.[12] However, the confirmation of HPV infection in the epithelium now creates additional possibilities for the nature of the deep cells.

I. THE DEEP CELLS ARE NON-DYSPLASTIC

It is possible that the morphologically disturbed deep cells are not destined for dysplastic behaviour, and this could perhaps explain the regression of some cervical dysplasias.[13] They may thus represent early non-koilocytic or pre-koilocytic stages of a productive infection that ultimately leads to koilocytosis and death of the maturing cells with a negligible risk of malignant transformation. When the infection declines, a return to normal appearances would be expected.

II. THE DEEP CELLS ARE DYSPLASTIC

Dysplasia in the deep cells could be either causally unrelated or causally related to HPV infection.

A. Dysplasia Not Causally Related to HPV

Dysplasia due to any cause might be established in the cervix before the onset of HPV infection. The association between koilocytes and disturbed deep cells might reflect merely the contingency in space and time of two quite unrelated states. Indeed, the relationship might be in the

opposite direction since dysplastic cells would be killed by koilocytosis and might regress.

B. Dysplasia Possibly Causally Related to HPV

Dysplasia due to HPV alone.—Here we postulate that the deep cells are dysplastic and that papilloma-virus infection is causative. In doing so we have to explain certain difficulties. The epithelium at first sight (figure b) appears to be undergoing a productive (koilocytic) infection, in which transformation of the koilocytes to a proliferative malignancy is impossible. Is it then possible that the deep cells may have a different non-permissive viral association? The question remains unanswered, but a plausible hypothesis and some findings support the possibility. With an invariant strain of oncogenic virus the establishment of a non-permissive rather than a permissive infection usually entails a significant change in the nature of the host cell—eg, a change of species. However, cervical squamous epithelium contains a broad spectrum of morphological and functional cellular differences, ranging from immature basal cells through actively mitotic parabasal cells to the rounded cells of the stratum spinosum and the flattened superficial glycogenated squames. Virions released from the productively infected koilocytes could find their way through the intercellular spaces to the deeper strata and could well strike up a non-permissive infection in them. The non-permissive association would be largely determined by the different nature of the deep cells, but mutations in the "productive" virions might assist. Transformation to dysplasia in multiple foci would be consistent with the common association between koilocytes and dysplasia. The epithelium might then be thought of as semi-permissive, with the unusual property that the distribution of permissive and non-permissive cells is not random as in a cell culture but topographically predictable, with koilocytes in the superficial strata and transformed dysplastic cells in the deeper zones. Our hypothesis that cervical epithelial cells of different degrees of differentiation may relate differently to virus is supported by studies suggesting that the nature of virus growth in cells can be altered by organ, or even functional, differentiation rather than by a change of species. Thus, susceptibility of mouse organs to polyoma infection was determined not only by organ differentiation (salivary gland or metanephros) but also by the metanephric epithelium, mesenchyme, and stage of development.[14] In SV-40 infection of murine teratocarcinoma cells undifferentiated cells were resistant but differentiated cells supported SV-40 non-permissively.[15] Stem cells transfected with a plasmid bearing SV-40 genome did not express early protein until induced to differentiate by retinoic acid.[16] In murine cytomegalovirus infection of teratocarcinoma cell lines differentiated cells were permissive but undifferentiated cells supported only a latent infection until induced to differentiate with dimethylacetamide, when viral production began.[17] In addition, purely functional differences in related morphologically similar cells have been shown to alter virus growth patterns, as with herpes-simplex infection of lymphoid cells maturing from the "null" state through the different stages of the divergent B and T cell paths to mature lymphocytes.[18]

Dysplasia due to viruses other than papilloma virus.—Herpes-simplex type-2 virus (HSV-2) has for long been linked by circumstantial evidence to cervical carcinoma. Inactivated virus can transform rodent cells to malignancy in vitro,[19] and antibodies against it[20] are found in higher titre and are more common in women with cervical cancer than in controls, although there is considerable geographical variation.[21] HSV-2-specific mRNA has been detected in premalignant and malignant cervical tissue.[22–24] Direct evidence for a causal connection between HSV-2 and cervical cancer has not been forthcoming, and HSV-specific DNA has only rarely been found in cervical malignancy even with highly sensitive hybridisation techniques.[25] HSV-2 as a sole cause of cervical cancer remains in great doubt. On the other hand, dysplasia brought about by HSV-2 in synergism with other agents, notably human papilloma virus, is plausible.

Viral synergism as a cause of dysplasia.—zur Hausen proposes a synergism between HSV and HPV which leads to cervical neoplasia.[26] The hypothesis resolves the conflict between the serological evidence favouring past HSV infections and the general failure to detect HSV-2 DNA in the neoplasms. It proposes that a transient cervical epithelial infection with HSV raises the antibody level and "initiates" the malignancy. Initiation of the cellular genetic change to neoplasia is not brought about directly by integration of HSV oncogene but indirectly by DNA repair enzymes transcribed from the virus early in the infection. The viral enzymes (deoxyribonuclease or a DNA polymerase with additional exonuclease activity) bring about chromosomal aberrations or gene amplification in the cellular genome—a known mechanism of physical and chemical carcinogensis. The cellular mutation is, of course, transmissible to daughter cells but need not be accompanied by virus—thus explaining the absence of HSV-2 DNA from the neoplasms. HSV thus acts as a "hit and run" type of initiator of neoplasia. zur Hausen further postulates that cells initiated by HSV could be promoted by a superimposed HPV infection to recognisable dysplasia.

Dysplasia due to synergism between virus and other carcinogens.—Synergism with papilloma or other viruses could provide the component necessary to give expression to some of the other risk factors of cervical carcinoma—eg, the tenuous association with smoking[27] or nitrosamines[28,29] or other substances so far undetermined. To test the hypotheses outlined above, it would be necessary to purify the virus from cervical koilocytes and to compare it with the 15 known types and several subtypes of HPV. As papilloma virions are probably present in very small numbers and cannot be cultured productively in vitro, the viral DNA could be cloned in a suitable vector, as has been achieved with bovine papillomavirus genomes.[30] Thereafter in-situ hybridisation experiments should reveal which cells of the koilocytic epithelium contain HPV nucleic acid, and perhaps shed light on what is happening in the deeper layers.

Correspondence should be addressed to S. F., Pathology Department, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG.

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DETECTION OF HUMAN PAPILLOMA VIRUSES OF THE GENITAL TRACT

Mary Norval

Department of Bacteriology

University of Edinburgh Medical School

Teviot Place

EDINBURGH EH8 9AG

CONTENTS

1. INTRODUCTION

Papilloma viruses are known to infect a variety of species including man, dogs, horses, rabbits, deer, sheep, cattle, mice and birds, and to induce papillomatosis (warts) in these animals (reviewed by Lancaster and Olson, 1982). Warts have been recognised for centuries and were experimentally transmitted almost eighty years ago (Ciuffo, 1907). Traditionally warts were thought of as benign or even hyperplastic lesions of cutaneous or mucosal tissues but gradually their potential for transformation to carcinomas was recognised. In particular, in recent years, an association between human papilloma viruses (HPV) and the development of genital neoplasias has been found, which has fostered interest in the papilloma viruses and their detection. Concomitantly, significant advances in the molecular cloning of papilloma viruses have partly compensated for our inability to culture the viruses in vitro and have made possible some studies on their behaviour.

In this chapter, the biology of HPV types is introduced followed by a review of genital warts and the association of HPV with genital carcinomas. Methods for the detection of HPV in genital lesions are then given and assessed. The relevance of monoclonal and polyclonal antibodies is discussed.

2. HUMAN PAPILLOMA VIRUSES

A. Papilloma virus types

Papilloma viruses belong to the genus A of the family Papovaviridae. The virions are naked, about 50 nm in diameter, with icosahedral symmetry and containing 72 capsomeres. Particles of HPV prepared from plantar warts by grinding skin scrapings followed by equilibrium centrifugation in CsCl are shown in Fig. 1.

In the past few years it has become clear that there are many types of papilloma viruses which infect particular species in preferential sites and with typical clinical manifestations. In man, the number of types has risen to 35 and more are still being discovered. Types 1 to 18 and their associated clinical lesions are outlined in Table I.

A new type is designated if it shares less than 50% sequence homology with other papilloma viruses as tested by reassociation kinetics under stringent conditions of nucleic acid hybridisation (Coggin and zur Hausen, 1979 ; see Section 6E below for an explanation of hybridisation conditions). There should also be significant antigenic variation from any other type although this is often not possible to test if virus particles are either not present or are present in low numbers. Furthermore, viral protein products are not well defined at the present time either in terms of molecular weight or function. In practice, typing is based on molecular cloning of viral DNA, followed by blot hybridisation with known human types under different stringency

conditions. A subtype has less than 100% but more than 50% sequence homology with known types. Perhaps it is surprising that typing without characterisation of viral mRNA and viral proteins should lead to such good concordance with clinical lesions. Recombination between different types has been suggested to occur (Gissmann, 1984).

B. Genomic organisation

The genome of HPV types consists of a double-stranded, covalently closed, circular DNA molecule containing about 7900 base pairs. As papilloma viruses cannot be cultured in vitro it is important to ascertain their genomic organisation from nucleotide sequence analysis so that predictions can be made about their possible protein products, control elements and associations with different types of cells.

Some HPV types are found in small numbers only in wart lesions, as in genital warts, and some are present only in the form of viral DNA, not as mature virus particles, as in cervical carcinomas. Thus sequencing in these cases depends on successful molecular cloning of papilloma virus DNA. Where possible, the cloning methods depend on the isolation and purification of viral DNA followed by linearisation either at the single BamHI or ECoRI site, making recombinant plasmids, often with pBR322, and cloning in suitable bacterial strains. Where viral particles are not found, a genomic library of the appropriate tissue is made by cleaving cellular DNA with a restriction endonuclease using bacteriophage λ as vector. Recombinant phages containing viral DNA are then cloned. These strategies are reviewed by Gissmann and Schwarz (1984).

Initial studies led to the elucidation of the nucleotide sequence of bovine papilloma virus type 1 (BPV 1, Chen et al, 1982) and subsequently the sequence of HPV 1a, (Danos et al, 1982; Clad et al, 1982), 6b (Schwarz et al, 1983) and 16

(Seedorf et al, 1985) have been found. The general organisation is very similar in all these types and is illustrated in Fig. 2 for HPV 6b and 16.

Intracellular replication of DNA viruses is often split into three phases: firstly, early proteins are synthesized which are predominantly non-structural in function, then viral DNA is replicated and finally late proteins are synthesized which are mostly structural, making up the capsomere sub-units of the virus particle. The proteins are coded for by open reading frames on the viral DNA. All significant open reading frames (ORFs) are on one DNA strand. These potentially encode polypeptides greater than 90 amino acids. There are several presumptive early ORFs, designated E1 to E7, on the basis of size, and two late ORFs, L1 and L2. Between L1 and E6 is a non-coding region of about 1000 base pairs which is thought to contain the origin of DNA replication and the promoter for early gene transcription. Transcriptional enhancer elements functioning in a position- and orientation-independent manner have been mapped within this region and also in the other region between the E and L parts of the genome (Spalholz et al, 1985; Lusky et al 1983).

An <u>in vitro</u> transforming system is available for BPV 1 and had yielded interesting results on genomic organisation. A segment containing 69% of the viral genome was found to be necessary for transformation of certain mouse cells to focus formation, growth in soft agar and tumorigenicity in athymic nude mice (Lowy et al, 1980); further, the genetic information encoded by E2, E3, E4 and E5 was sufficient to induce

transformation (Yang et al, 1985a; Lusky and Botchan, 1985). E1 function is involved in extrachromosomal viral DNA maintenance in transformed cells (Lusky and Botchan, 1984). E2 is responsible for the transactivation of the enhancer located in the non-coding region (Yang et al, 1985b). This could lead to a direct effect on transformation as well as increased expression of other ORFs including E6, E7 and E1. E5 may be involved in inducing proliferation of dermal fibroblasts in fibropapillomas. It will be interesting to see if the early ORFs of HPV have similar functions, despite their lack of homology with BPV 1 and their biological differences in inducing epithelial proliferation rather than fibropapillomas.

Differences in coding potential between different HPV types may be of functional significance. For example, HPV 16 which is associated mainly with genital carcinomas, is not like HPV 1a and 6b in some respects. The ORFs for E1 of HPV 16 is split into 2 reading frames, and E4 and E5 do not have a translation initiation codon (Seedorf et al, 1985). The importance of these changes is not known at the present time. By studying HPV 16 and 18 sequences in various cervical cell lines derived from carcinomas, the presence of E1 but probably not E2 was thought to be necessary for expression of the malignant phenotype (Pater and Pater, 1985).

Viral mRNA synthesis has been examined mainly in BPV 1 and cotton tail rabbit papilloma virus (CRPV) systems (reviewed by Pfister, 1984). Some interesting results with CRPV show that transcription differs in virus-producing benign tumours from

malignant non-productive tumours (Phelp et al, 1985). The regions of the genome containing the ORFs, once transcribed to RNA, probably undergo excision and splicing to make the mRNAs. Work has begun on analysing viral mRNAs in cervical cell lines to find out which regions of the genome are transcribed, whether or not splicing occurs and whether co-transcribed host sequences are important (Schwarz et al, 1985). In one report, HPV 16 mRNA was found only rarely in cervical carcinoma biopsies containing HPV 16 DNA (Lehn et al, 1985). Thus continuous expression of HPV may not be necessary for the maintenance of a transformed state. The implications for detection methods, such as assaying for HPV protein products are obvious.

C. Viral protein products

To date, none of the early gene products of HPV types have been identified in in vitro transforming systems or in warts, and no specific tumour antigens are characterised in vivo. It is likely that such proteins are, in fact, synthesized as shown by the conservation of the amino acid sequences coded for by the early regions of different papilloma virus genomes. However they may be present only in small quantities or be weakly antigenic so that new methods may have to be developed to detect them. One approach may be to translate viral mRNAs in vitro. Another may be to take segments of ORFs, especially the early ORFs, and to insert these into bacterial expression vectors under the control of inducible promoters, in the hope of getting translation of viral products.

L1 and L2 are expected to code for the viral structural polypeptides. Major polypeptides of molecular weight 57,000, 54,000 and 44,000 have been described in purified HPV 1, 2 and 3, in association with other minor polypeptides of smaller molecular weights and which varied between isolates (Gissmann et al, 1977). Such work has been dogged by difficulties in the preparation of adequately purified virus particles from pooled clinical material and in sufficient quantity for analysis. However it should be possible to obtain a sufficient quantity for the preparation of monoclonal antibodies. In BPV 2, L1 codes for the major polypeptide of the capsid which has molecular weight 55,000; L2 probably codes for a polypeptide of molecular weight 50,000

(Potter and Meinke, 1985).

Antisera have been prepared against purified HPV, where feasible, i.e. types 1, 2, 3, 5, 8 and 9 (Jablonska et al 1982; Gissmann et al, 1977). These are type-specific and detect viral capsid antigens in the nuclei of infected cells. If the antisera are prepared using virus particles disrupted with sodium dodecyl sulphate (SDS) then the type specificity is lost and the antisera cross react, not only with the capsid antigens of other HPV types, but also with papilloma viruses from other species, such as cattle, dogs, horses and deer (Jenson et al, 1980). Again the antigen is located in the nuclei of infected cells. Probably there are amino acid sequences shared between all papilloma viruses which lie in a part of the capsid protein not normally exposed on the surface of virions, which then account for the genus-specific reactivity. As will be outlined in Section 6D, both the genus-specific and type-specific antisera to the capsid antigens are of use in screening tissues for evidence of papilloma virus infection.

3. HUMAN WARTS

Wart lesions induced by HPV infection are primarily epithelial proliferations of the skin or mucosa. This results in overgrowth of sub-epithelial capillaries (papillomatosis), and in thickening of the stratum spinosum (acanthosis) and stratum corneum (hyperkeratosis). Eventually, probably to accomodate the epithelial proliferation on a limited base, the basement membrane and the overlying strata corrugate creating strands or outgrowths whose vessels (which grow correspondingly) nourish the epithelium. The lesion is thus essentially fibro-epithelial with, usually, some (limited) branching of the processes or "asperities".

Warts are benign, in most instances, showing limited growth and generally regress spontaneously after several weeks or months. They can persist, however, and can recur. Abrasion to the skin is probably required before the virus is able to infect a new host and the incubation period is thought to be between 3 and 18 months (Rowson and Mahy, 1967). It is not known what is happening during this period, how infective HPV types are and whether infections are sometimes sub-clinical.

A few cells of the basal layer contain HPV DNA in their nuclei (Beckmann et al, 1985) and many more in the first or second suprabasal layer of the stratum corneum (Grussendorf and zur Hausen, 1979). The viral DNA may be integrated or extra-chromosomal, although the latter is the more commonly found state with most, but not all, types. The parabasal cells

probably represent the first target for the virus. The structural proteins begin to appear in the cells of the upper layers of the stratum spinosum and virus particles, when present, in this layer and the stratum granulosum. The virions can sometimes be seen in paracrystalline arrays in the nuclei and enmeshed in keratin in the stratum corneum. Fig. 3 shows a diagram of the epidermis infected with HPV, and Fig. 4 an array of HPV 1 in the stratum granulosum of a plantar wart.

The cells of the basal and suprabasal layers may be non-permissive for HPV and the association with virus a transforming one (Fletcher and Norval, 1983). In vitro HPV fails to replicate productively even in cultures of keratinocytes which are forming a granular layer and is found only as viral DNA, 50 to 250 copies per cell, present as stable non-integrated genomes (Taichman et al, 1983). As the keratinocytes become more differentiated in the stratum spinosum, the association with virus probably becomes a permissive one. The productive infection in the superficial cells is marked by the presence of koilocytic cells, first noted by Koss and Durfee (1956). In brief, these can be described as having densely staining, irregular,indented or folded nuclei and a ballooned cytoplasm with a clear, glycogen-free perinuclear halo. Sometimes there are basophilic nuclear inclusions which consist of arrays of virus particles. The koilocytes are considered pathogonomic of HPV infection. Different types of koilocytes have been described by their histopathological appearance (Crum et al, 1985) but whether these represent separate clinical entities, infection

with different HPV types or stages or permissiveness for HPV replication is not known.

There is increasing evidence that HPV can become latent and may be present in the epidermis without clinical lesions and morphological signs. The cervix, in particular, may be subject to latency which may be significant for the diagnosis and management of patients (Ferenczy et al, 1985; Syrjanen et al, 1985).

4. GENITAL WARTS

It has become increasingly evident that papilloma viruses associated with infections of the male and female genital areas are spread predominantly by sexual transmission. There is probably no correlation between genital and skin warts. Genital warts are found in populations of high promiscuity (Waugh, 1972), in age groups of sexual maturity and where sexual activity is highest (zur Hausen, 1977). The sites of genital warts are commonly those of frequent contact during coitus. Horizontal spread can be traced from subjects with the disease (Oriel, 1971). The incidence of genital HPV infection increased during the last decade and now represents at least 6% of all cases seen in Genito-Urinary Medical Departments in Britain (Adler, 1984). In Edinburgh 13% of male patients (out of 5,013) and 13% of female patients (out of 3,230) attended the Genito-Urinary Medical Department with genital warts in 1984 (A. McMillan; personal communication).

In men, genital warts are found on the glans and body of the penis, prepuce, fraenum and coronal sulcus, urethral meatus, scrotum, anus and rectum. In women, they are present on the introitus, vulva, vagina, cervix, perineum, anus and rectum. Until 1976, only one type of genital warts was described, the condyloma acuminatum, but now several others are recognised, the most important being the flat condyloma and Bowenoid papulosis.

A. Condyloma acuminatum

The classical exophytic genital wart or condyloma acuminatum

is characterised by papillomatosis, acanthosis, elongation and thickening of the rete pegs and koilocytosis (Woodruff and Peterson, 1958). Its growth pattern is illustrated in Fig. 5a and a clinical example in Fig. 6b. It is found on the penis, anus, vulva, perineum and cervix (zur Hausen, 1977). Virus particles are seen in some lesions, around 50% in most surveys, although not normally in high numbers. Viral antigens have also been detected and viral DNA, predominantly types 6 and 11 (Gissmann et al, 1983). Malignant conversion into carcinomas of the penis and vulva is rare (zur Hausen, 1977).

Giant condylomata located on the penis were described by Buschke and Lowenstein in 1931. They have invasive growth properties after long periods of time but without metastases. They are associated with HPV 6 and 11 (Zachow et al, 1982).

B. Flat condyloma (flat koilocytosis)

In the cervix, predominantly, HPV infections assume a less exophytic form and flat lesions are found. They appear often as white epithelium in the transformation zone of cervical ectropion where subcolumnar reserve cells are proliferating and re-differentiating into squamous epithelium. It is not known why there should be a predilicted site for this type of wart, but perhaps the rate of cell or tumour production is highest here, or the keratin pattern may be unusual. Flat condylomata were only recognised as being wart lesions in 1976 (Miesels and Fortin, 1976) and, on colposcopy, are often very difficult to distinguish from lesions of cervical intra-epithelial neoplasia (CIN), being classified traditionally as CIN grade 1 (mild dysplasia). They

are also found in the vulva and penile glans (Gross et al, 1985b). The growth pattern of flat condyloma is shown in Fig. 5b, and a clinical lesion in the cervix in Fig. 6c. The nomenclature of this type of wart is confusing as it has been called variously a flat wart, a non-condylomatous cervical wart, subclinical HPV, flat koilocytic lesion and atypical condyloma, but the term flat condyloma is being used increasingly despite the inherent contradiction in this description (condyloma is derived from Greek kondylos : knuckle; see Fletcher, 1983, for a critical review of nomenclature and classification).

In this decade flat condylomata of the uterine cervix have become recognised as common infections. It has been estimated that 1.3% of unselected women show cytological changes consistent with HPV infections on routine Papanicolaou smears and 25% of colposcopically directed punch biopsies also show similar changes (Reid et al, 1980). There is evidence to suggest that the incidence of flat condylomata of the cervix is increasing although there is more awareness of this condition and better detection methods than formerly (Oriel, 1983). As with condylomata acuminata, flat condylomata show koilocytic cells on histology; genus-specific viral antigen is present in about half the cases although its distribution is often patchy and sparse compared to exophytic genital warts (Woodruff et al, 1980). HPV 6 and 11 DNA are found in 50-60% flat condylomata (Gissmann et al, 1982; Gross et al, 1985b).

In addition to the flat condyloma, an endophytic or inverted condyloma has been described (Meisels et al, 1976). Here

squamous epithelium has proliferated in the necks of the endocervical glands with superficial koilocytosis. It is possible that this is merely a papilloma virus infection of a branching epidermidised gland and Fletcher (1983) has suggested that it should not be considered as different from flat condyloma.

C. Bowenoid papulosis

Classically this is described as flat, multifocal, slightly pigmented papules, often verrucoid, in the body of the penis, first noted in 1970 (Lloyd, 1970) and affecting young adults. Although appearing to be benign macroscopically and often regressing spontaneously, these papules show histological changes of squamous cell carcinomas-in-situ. They assume a flat, papillary endophytic or papillomatous exophytic form (Gross et al, 1985). Only 5% of lesions show the genus-specific antigen of HPV but over 50% contain HPV 16 DNA (Ikenberg et al, 1983; Braun et al, 1983; Gross et al, 1985a). This is taken to indicate low productive infection with HPV 16 in epithelial cells predominantly non-permissive for the virus and transformed to some extent by the presence of viral DNA. Bowenoid papulosis of men may represent an important reservoir of HPV 16.

Genital Bowen's disease is found histologically to be very similar to Bowenoid papulosis except lesions are not multifocal and they occur on the glans of the penis (Wade, 1978). The average age of patients is 50, and HPV 16 is found in 80% cases (Ikenberg et al, 1983).

5. ASSOCIATION OF HPV WITH GENITAL CARCINOMAS

Almost 150 years ago Rigoni-Stern (1842) observed that uterine cancers were more prevalent in married than in unmarried women, and were almost absent in certain orders of nuns. Confirmation of these findings indicating that sexual behaviour and cervical cancer were associated was published in 1973 (Rotkin), and since then a sexually transmitted agent has been sought. In addition, based on epidemiological data, a progression from a pre-malignant change in the cervix to a truly malignant state is suggested (Meisels et al, 1982). These stages are recorded histologically as CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia), CIN 3 (severe dysplasia/carcinoma-in-situ) and invasive carcinoma. Various viruses such as herpes simplex type 2 and cytomegalovirus have been implicated as possible agents in this neoplastic process and HPV suggested only a few years ago (zur Hausen, 1976). However the association between HPV and abnormalities of the uterine cervix gained ground rapidly after it was realised that CIN often occurred in conjunction with koilocytosis (Meisels et al, 1982).

Various groups soon added data in support of HPV infections being important in CIN. Most notably, techniques have included histopathology looking for features of papilloma virus infection, screening for HPV antigen using an antiserum against the genus-specific papilloma antigen, and detection of HPV DNA by nucleic acid hybridisation. Some selected results and references are summarised in Table 2. In addition to this

data HPV 16 is found in CIN lesions where there are abnormal mitotic figures and nuclear atypia in all layers of the squamous epithelium. These lesions have an aneuploid nuclear DNA content and a high risk of progression to malignancy (Crum et al, 1984; Crum et al, 1985).

Similar results to that found in the cervix have been obtained for vulval condylomata and vulval intra-epithelial neoplasia (VIN) (Boshart et al, 1984; Gissmann, 1984; Ferenczy et al, 1985).

While the viral DNA in HPV 6 and 11 lesions is in the same state as in other papilloma virus infections, i.e. non-integrated and circular (Lehn et al, 1984), that of types 16 and 18 in carcinomas may be different. There is evidence for head-to-tail tandem repeats persisting in a non-integrated and circular form, and some integrated genomes as monomers or as head-to-tail repeated oligomers, up to 20 per integration site (Boshart et al, 1984; Durst et al, 1985). In another study HPV 16 DNA was found as a single copy co-valently linked to tumour cell DNA in a cervical carcinoma (Lehn et al, 1985). Integration of HPV 16 and 18 also occurs in some cervical cell lines (Schwarz et al, 1985; Pater and Pater, 1985; Yee et al, 1985). In contrast, one group failed to find HPV DNA sequences in the majority of invasive squamous cell carcinomas of the cervix (Fukushima et al, 1985). The sensitivity of the assay system used was estimated at one copy of viral genome per 20 cells with a homologous HPV probe and one copy per three cells with a heterologous DNA probe.

From all this data, the consensus view is that HPV 6 and 11

are found most commonly in genital warts and in CIN and VIN of low grades in an non-integrated form and that this association is one of low malignant potential; on the other hand, HPV 16 and 18 are found most commonly in genital carcinomas in an integrated form and have high malignant potential (Crawford, 1984). This hypothesis remains to be fully tested and it is unlikely to be the whole story. Various co-factors are undoubtably important as suggested by zur Hausen (1982), such as infection with a second virus or bacteria or even smoking. So far it has been impossible to document if HPV plays a casual or causal role in genital neoplasias (Singer et al, 1984) and more data is required, particularly on what happens during progression of the lesions.

6. DETECTION OF HPV GENITAL INFECTIONS

These methods are wide-ranging and include gross appearance, colposcopy, histopathology of cervical smears and biopsies, electron microscopy, detection of HPV antigens, detection of HPV DNA and immune responses. Each test gives valuable information although the only reliable method of typing HPV at the present time is by DNA hybridisation. Most reports combine more than one technique which, perhaps, is the best policy until the position becomes clearer and definitive reagents are available.

A. Gross appearance and colposcopy

There is little problem in identifying the classical condyloma acuminatum by its gross appearance and a good description is given in Woodruff et al (1958). On colposcopic examination of the vagina and cervix, there is often a thick white epithelial focus with surface projections containing capillary loops. Early lesions are more difficult to define and have only small surface projections (asperities) (Meisels et al, 1979; Reid et al, 1980).

Flat condylomata have a smooth or irregular surface, only slightly raised from the surrounding epithelium. They exist in both dysplastic and non-dysplastic forms. Recently, the colposcopic appearances of HPV lesions in the cervix have been classified as normal, mosaic, warty, leukoplakial (thickened white epithelium) or a combination (Vatyrynen et al, 1985). However, colposcopic approaches do not reliably detect and separate the types and, in practice, clinicians are more

concerned about dysplastic zones, whether koilocytic or not, and tend to direct their attention and their biopsies to them. Distinguishing patches of dysplasia from non-dysplastic flat condylomata or co-incident dysplasia and koilocytosis is unreliable colposcopically and requires histological examination (Kirkup et al, 1982). Condylomata can be suspected if the lesions are multiple, if they are present outside the transformation zone and if there are dilated capillaries in the surrounding native squamous epithelium (Meisels et al, 1977).

B. Cytology and Histopathology

There are characteristic cellular changes in Papanicolaou smears of women with condylomata (reviewed by Meisels et al, 1984, and Syrjanen, 1984). Normally no inflammatory cells are present, but koilocytes and dyskeratotic cells are, although not invariably. The koilocyte is considered pathogonomic for HPV infection. It is an intermediate or superficial squamous cell with enlarged hyperchromatic nucleus and an area of perinuclear clearing (see Fig. 7). The rest of the cytoplasm is thickened, often with an amphophilic staining pattern. It may be bi- or multi-nucleated. Dyskeratocytes are often shed singly or in clusters. They are small keratinised squamous cells with orangeophilic cytoplasm and large homogeneous nuclei. Sometimes the nuclei are atypical and it is difficult to distinguish papilloma virus infections from CIN or carcinoma in such cases. Neoplastic cells have a well-preserved chromatin structure, defined nuclear membranes and abnormal nucleo-cytoplasmic ratios but these features are hard to define and sometimes characteristic

cells are not shed from the condyloma. Colposcopically directed cervical biopsies should be taken where there is an abnormal Papanicolaou smear.

Some features of the histopathology of genital warts have already been covered in Section 4. Condyloma acuminatum is distinguished by papillomatous growth with acanthosis. There is elongation and thickening of rete pegs and koilocytes are prominent, often in the tips of the papillary projections. In the flat condyloma, the most striking feature is the presence of koilocytes in the middle and upper epithelial layers.

In some cases, the flat condylomata are found in association with CIN, either within the pre-malignant lesion or adjacent to it (see Fig. 8). This makes the detection of HPV infection and the grading of the dysplasia very difficult. Fletcher (1983) suggests a simplified and helpful scheme of reporting on the histopathology of cervical biopsies which is to grade the dysplasia with and without koilocytosis and, if desired, to note whether the koilocytes are adjacent to, remote from, or co-incident with the dysplasia.

An attempt has been made recently to clarify this area and to forecast which lesions might progress by reporting on the ploidy pattern of biopsies (Fu et al, 1983). While diploidy represents a normal cellular pattern, polyploidy is indicative of hyperplasia and benign neoplasms, and aneuploidy of a malignancy (Bohm and Sandritter, 1975). It is possible to quantitate nuclear DNA in smears or tissue sections by microspectrophotometry or computerised image analysis after Feulgen staining.

In condylomata, diploid or polyploid DNA is found, while in CIN, persisting for a year or more, aneuploid DNA is detected; in CIN which regressed, most nuclei are diploid or polyploid. This technique is demanding and time-consuming, perhaps not likely to be useful in a routine laboratory, but automated fluorescent flow cytometry may be developed in the near future to give similar information.

Measurement of DNA content of cells has been complemented by observations of abnormal mitotic figures and diffuse nuclear atypia in VIN lesions (Crum et al, 1982). In vulval condylomata, nuclear atypia is seen in the mature epithelial cells only and abnormal mitoses are absent. Furthermore last year Crum et al (1985) showed that a distinction between HPV types in CIN might be made histologically. Condylomatous lesions containing koilocytic atypia and nuclear atypia in the upper epithelial layers were infected with HPV 6 or 11 in 61% of cases. CIN lesions in association with condylomata showing nuclear atypia in all the epithelial layers and/or abnormal mitoses plus koilocytic atypia were infected with HPV 16 in 82% of cases. CIN lesions with minimal epithelial layer maturation and nuclear atypia in all epithelial layers and/or abnormal mitoses had evidence of HPV 16 DNA in 33% of cases and untypable viral DNA in 22% of cases. This analysis demonstrates that very careful histopathology may be able to pinpoint lesions containing HPV 16 and which may have a tendency to progress. However the CIN lesions of grades 1 and 2 in association with HPV infections may be difficult to fit into this histological categorisation.

Furthermore, in contrast to the results of Crum et al (1985) another study of HPV infections of condylomatous and condylomata-like lesions of the male and female genital tract, perianal skin and anal mucosa, concluded that histopathology was not sufficient to predict HPV type, even taking details of the koilocytic features into account (Gross et al, 1985b). The only correlation found was between Bowenoid papulosis lesions featuring severe epithelial atypia and no koilocytic cells which contained HPV 16 DNA.

At the present time, therefore, it seems wise to substantiate the histopathological appearance of condylomatous or dysplastic lesions with detection of HPV by additional methods. However, from the point of view of the patient, a very useful grading of cervical dysplasias can be made using rapid frozen sections which avoids the delay inevitable with paraffin sections. The patient can therefore be advised at the time of colposcopy and treated immediately after diagnosis (Fletcher et al, 1985).

C. Electron microscopy

Condylomata acuminata prepared for electron microscopy by standard procedures were first shown to contain HPV virions in 1968 in Edinburgh (Dunn and Ogilvie, 1968). The virus particles have characteristic size and shape which make them relatively easy to detect in cells, even at low densities. Since then several groups have undertaken surveys of flat condylomata and reported HPV in approximately half the biopsies screened (Reid et al, 1980; Pilotti et al, 1981; Ferenczy et al, 1981). The

particles are always found within the nuclei of koilocytic cells and sometimes in the superficial dyskeratotic cells but, in all types of genital warts, are present in low numbers only. HPV particles have never been reported in malignant squamous cell tumours (Syrjanen and Pyrhonen 1982; Kurman et al, 1981).

It is possible to reprocess cells for electron microscopy from Papanicolaou smears using the methods outlined in Smith and Coleman (1983). Virus particles are seen in koilocytes and dyskeratocytes in about half the smears examined which showed overall cytological evidence of HPV infection (Meisels et al, 1984).

While electron microscopy has provided valuable information about productive HPV infections in genital condylomata, it is a painstaking technique given the paucity of virus particles. It will not distinguish different types of HPV and obviously cannot be used to detect HPV DNA in malignant tumours. Apart from research purposes, it has been superceded, largely, by detection of HPV antigens (see Section 6D). However, recently, interesting results have been obtained on the possible latency of HPV in the cervix by using electron microscopy. Syrjanen et al (1985) screened punch biopsies by electron microscopy in a prospective follow-up of women who had had abnormal smears, and reported finding HPV particles in tissues which were colposcopically and histologically normal. Although this work could be done by DNA hybridisation methods as outlined in another study (Ferenczy et al, 1985), information on the morphology and numbers of virions and cell types infected can be obtained only from electron

microscopy. Thus the electron microscope provides useful additional, and sometimes, thought-provoking results.

D. Detection of HPV antigens

The method commonly employed for the detection of HPV antigens in genital condylomata is peroxidase-antiperoxidase as outlined by Sternberger (1979; reviewed in Meisels et al, 1984). This procedure has the great advantage that sections of formalin-fixed, paraffin-embedded material can be used. An antiserum with papilloma virus genus-specificity is made by injecting rabbits with purified disrupted HPV 1 from pooled plantar warts or BPV 1 from bovine fibropapillomas. This antiserum binds to viral antigens and is detected by adding a secondary antiserum followed by a soluble complex of peroxidase-antiperoxidase which binds to the free site of the secondary antibody. A substrate of peroxidase and H_2O_2 are added; in a positive test, a dark brown staining results.

In essence, HPV antigen is detected in the nuclei of some koilocytes and occasionally in dyskeratocytes. In some surveys it is found more often in flat condylomata than in exophytic lesions (Morin et al, 1981; Ferenczy et al, 1981), while in others the reverse is found (Syrjanen et al, 1983). The degree of positive staining is always variable although it is most marked where the koilocytosis is most extensive, and it ranges from abundant, patchy and sparse, to negative. Even examining adjacent epithelial asperities in condylomata acuminata showed great variation in staining for no known reason (Woodruff et al, 1980). Syrjanen et al (1985) found no correlation between the

presence of HPV particles seen on electron microscopy and the presence of viral antigens, and surmised that the genus-specific antigen may not necessarily be expressed or exposed at the same time as virus particles are found.

With regard to CIN and VIN, positive staining is found to be inversely related to the degree of dysplasia and is not found, generally, in carcinomas (Reid et al, 1982; Kurman et al, 1983; Crum et al, 1982).

Variations of the peroxidase-antiperoxidase method include using biotinylated secondary antibodies with a detection system of avidin and biotinylated horse-radish peroxidase. In a study by Gupta et al (1983), this technique was employed to compare positive staining for HPV antigens in Papanicolaou smears as compared with cervical biopsies; HPV antigens were detected in 67% of smears and 62% of biopsies. Thus cervical smears may be just as effective a starting material for detection of HPV antigens as biopsies.

At the present time, as outlined in Section 2C, no antibodies are available which can differentiate the genital HPV types. It would be extremely useful to develop such antibodies, either monoclonal or polyclonal, especially directed against early viral products. Attempts are being made in this direction and, now that the nucleotide sequences of HPV 1a, 6b and 16 are known, it may be possible to create artificial oligo-peptides with type specificity against which antibodies could be produced.

Recently Gorra et al (1985) characterised several monoclonal antibodies to BPV 1 which could distinguish lesions induced by

BPV 1 from BPV 2 and HPV 1, as tested by avidin-biotin immunoperoxidase staining. BPV 1 was purified from bovine fibro-papilloma, disrupted with SDS, and used to immunise mice. Sera from the mice were tested for antibodies to papilloma virus by immunofluoresence on acetone-fixed sections of bovine fibro-papillomas. Mice with high antibody titres were killed and their spleen cells fused with a non-immunoglobulin secreting myeloma cell line. Hybridomas were screened for immunoglobulin production, then by immunofluoresence for anti-BPV antibodies. Any positive cultures were cloned and further tested for type specificity. Three were found with type specificity for BPV 1, all of the IgG2a sub-class. It should be possible to make similar monoclonal antibodies against the HPV types present in large numbers in warts such as HPV 1, but it would be difficult to collect sufficient purified particles in the case of genital warts. As there is no expression of the genus-specific antigen in malignant genital tumours, monoclonals with type specificity for early viral proteins may be necessary to detect and type any HPV present in such a lesion.

Thus screening for HPV antigens using existing antibody preparations are of use in detecting HPV productive infections, but great advances would be made if antibodies were available of either type specificity or directed against antigens expressed in transformed cells.

E. Detection of HPV DNA by hybridisation

This approach is the only one to date which leads to typing of HPV. Furthermore the state of viral DNA (whether integrated

or not, whether a single copy or repeated and in what manner) within the infected cell or tumour can be ascertained. Hybridisation depends on single-stranded nucleic acids forming stable duplexes under appropriate conditions if their base sequences are sufficiently complementary. If double-stranded nucleic acid is heated, it denatures to single strands. The temperature at which half the double strands become single-stranded is called the T_M, and it can be calculated for any given nucleic acid:

$$T_M = 81.5 + 0.50\ (\%\ G+C) + 16.6\ (\log Na^+) - 0.60\%\ \text{formamide}.$$

It depends on the G + C content of the nucleic acid, the monovalent ion concentration and the concentration of denaturing agents like formamide.

The optimal temperature for duplex formation is 25°C below T_M. At this point (high stringency) only single strands with a high degree of complementarity remain as stable duplexes; if the hybridisation temperature is lower (low stringency) then less well matched duplexes remain together. These differential hybridisation conditions have proved very useful in HPV studies. Thus it is possible, first, to screen DNA from a lesion at low stringency with an HPV DNA as probe to look for any evidence of HPV DNA; then, if present, the conditions of stringency can be increased with use of different types of HPV DNA as probes, and this will specifically type the HPV DNA in the lesion (Bornkamm et al, 1983). A temperature of T_M-18°C has been used for stringent hybridisation, and a temperature of T_M-40°C for non-stringent (Ikenberg et al, 1983; Boshart et al, 1984).

Methods of hybridisation include Southern blotting, adaptations of dot blotting and in situ hybridisation.

In general terms, in Southern blotting, the cellular DNA from a biopsy is extracted and cleaved with one of several restriction endonucleases, ECoRI and BamHI being used commonly. The digests are run on agarose gels, denatured and transferred to nitrocellulose filters (Southern, 1975). The filters are incubated under the appropriate hybridisation conditions with cloned HPV DNA, labelled in vitro by nick translation, usually with ^{32}P. Molecularly cloned HPV DNA, commonly using the plasmid vector pBR322 in E. coli K12, is available for most HPV types (de Villiers et al, 1981). After hybridisation, the filters are washed under appropriate stringency conditions and autoradiography carried out. To calibrate the system, different genome equivalents of unlabelled HPV DNA are included in place of the cellular DNA; the sensitivity is of the order of 0.5 genome equivalents per cell. An analysis of condylomata acuminata for HPV 6 DNA by this method is outlined in Gissman et al (1982) and the detection of HPV 18 in cervical carcinomas in Boshart et al (1984).

Whether the viral DNA is integrated or not can be determined by a combination of methods (Durst et al, 1985). Firstly cellular DNA is centrifuged in a CsCl/ethidium bromide gradient which allows a distinction to be made between supercoiled, nicked-circular and linear DNA or DNA co-migrating with cellular DNA. Secondly the cellular DNA is analysed by 2-D gel electrophoresis after digesting with a restriction endonuclease

not cleaving the viral DNA. Following Southern blotting and hybridisation under stringent conditions, a linear (integrated) and circular (non-integrated) can be distinguished. Finally junction fragments between viral and cellular DNA can be detected after digestion of cellular DNA with a restriction endonuclease cleaving at several sites within the viral genome, followed by gel electrophoresis and hybridisation. The cleavage pattern is altered if the viral DNA is integrated. The DNA encompassing the junctions between the viral and cellular genome may be cloned and used to analyse integration sites and DNA from other tumours.

Dot blotting has been tried by several groups recently, mostly in attempts to use cervical scrapes for typing HPV without the need for cervical biopsies. The clinical procedure is no more demanding than collecting routine Papanicolaou smears. In one study (Schneider et al, 1985) smears were taken with swabs from the vagina, vulva, endo-and ecto-cervix, and the cells suspended by mixing in buffer before filtering directly on to nitrocellulose membranes for hybridisation without first extracting cellular DNA. 100,000 - 500,000 cells were obtained. Hydrodynamic filtration was tried by placing a small cup on the abnormal area of the cervix and irrigating the surface. The same results were obtained with both methods and showed good correlation with Southern blotting and histology. Sometimes infection with more than one type of HPV was detected which may reflect the relative cell populations obtained from smears and biopsies, and the relative sensitivities of dot blotting and Southern blotting. HPV DNA was largely confined to the tissue

seen to be colposcopically and cytologically abnormal but occasionally was found in normal tissue. It is not known if this result is due to latent HPV infections or to "false-positive" hybridisations. Follow-up studies are required.

Another group also used cervical scrapes but the cellular DNA in this case was extracted, put on gels, and transferred to robust Zetaprobe nylon filters before hybridisation (Wickenden et al, 1985). These filters can be stored and are re-hybridisable. A check was made on the quantity of DNA extracted from the scrape by hybridisation with an alu-repeat sequence probe. This particular sequence is found 5×10^5 times in human genomes, and 16% of samples had insufficient DNA. Only HPV 6 DNA was used as a probe in this study and it will be interesting to obtain data using a wider range of HPV DNA types.

Dot blotting has to be carefully controlled to ensure that the probe does not bind non-specifically to the filter. The probes are usually labelled with ^{32}P. This makes the procedure fairly slow (autoradiography requires 1 to 6 days for adequate exposure) and labour-intensive. Great efforts are being made to label the probe with a substance other than a radioactive tag, such as a fluorochrome or biotin (see below). This would eliminate the need for radioactive substances, enable batches of probes to be synthesised which would be stable, and reduce the time needed to detect duplex formation.

In situ hybridisation has proved a powerful tool in locating viral DNA or mRNA in specific cells from tumours and other tissues. It has the advantage over other hybridisation methods

of locating specific cells within a lesion which contain viral information. In addition paraffin sections prepared for routine histopathology can be used. Grussendorf and zur Hausen (1979) were the first to show which cells in plantar and finger warts contained HPV DNA. ^{3}H-UTP was used to label the cRNA of HPV 1 and the sensitivity was of the order of 20 viral genomes/cell. A similar study recently located HPV 16 DNA in nuclei of tumour cells from cervical carcinomas (Grussendorf-Conen et al, 1985). The viral DNA was labelled by nick translation with ^{3}H-TTP. Tritium-labelled probes need a long exposure time on autoradiography but produce contained, localised grains after development. ^{35}S-labelled viral probes are being tried which, theoretically, could produce localised grains on autoradiography in a much reduced exposure time. However high background labelling is a problem.

HPV DNA has been labelled with biotin *in vitro* instead of with a radioactive substance. Condylomata acuminata were then screened by in situ hybridisation (Beckmann et al, 1985). Hybridisation was carried out under stringent and non-stringent conditions using HPV 1, 6, 11 and 16 DNAs as probes. After hybridisation avidin was added, followed by goat antiavidin, biotinylated rabbit antigoat IgG, avidin-biotinylated peroxidase and finally a substrate for peroxidase. Each of these steps amplifies the sensitivity of the method which was estimated at 50-100 viral copies/cell genome using a reconstruction dot blotting experiment on nitrocellulose membranes.

At the moment, the sensitivity of hybridisation using

biotinylated probes is about a hundred times less than using radioactive probes. However the method is an attractive one and, hopefully, improvements such as synthesising more suitable biotinylated nucleotides and including more amplification steps will improve the situation.

F. Immune response

There is little information available on the immune response to HPV infections except it is generally agreed that circulating antibody titre is not a significant factor in predicting regression or persistence of warts, or re-infection (Cubie, 1972; Pfister and zur Hausen, 1978). However more sensitive methods for measuring antibodies have been developed in the past few years such as enzyme-linked immunosorbent assays (ELISA). One study with disrupted BPV 1 as antigen and an ELISA test has shown that IgG antibodies to papilloma antigens were present in 95% of patients with anogenital warts, 60% with CIN and 93% with cervical carcinomas. IgM antibodies were present in 54% of patients with anogenital warts, 29% with CIN and 95% with cervical carcinomas. Controls of children and adults without cervical lesions were never positive (Baird, 1985). It is hard to understand the significance of this data, both for the IgM and the IgG results. Some corroboration is required before such a test would be of use as a detection method for cervical abnormalities.

Cell-mediated immune responses are thought to play a role in regression of warts, with inflammatory cell exudates being characteristically found (Tagami et al, 1977). It has been

suggested that the initial cells of the wart may not be recognised by immune effector cells, perhaps because the products of the virally infected cells are immunosuppressive locally (Kirchner, 1984). Recently it has proved possible to examine wart and CIN lesions from the cervix for the distribution of such effector cells. Specific monoclonal antibodies were used which differentiate B lymphocytes, T cells of the helper and cytotoxic/suppressor subsets and Langerhans cells. Langerhans cells and T lymphocytes were much depleted in wart epithelium compared to normal epithelium, while there was an increase in Langerhans cells and T cytotoxic/suppressor cells together with increased stromal lymphocytes in CIN 3 (Morris et al, 1983). The Langerhans cells of the epidermis are known to be very important in recognition and processing of exogenous antigens within local sites, presenting antigens to the T lymphocytes and thus playing a crucial role during viral infection of the skin and in tumour induction (Wolff and Stingl, 1983).

Another study has characterised mononuclear cell infiltrates in cervical biopsies of women followed for at least 12 months (Syrjanen et al, 1984). No differences were found in the percentage of T cell subsets, B cells or mononuclear phagocytes between lesions which remained stationary, regressed or progressed. Progression was only related to the degree of CIN although there was a marked reduction in Langerhans cells in lesions which progressed compared to those which regressed or remained stationary. The significance of this finding is not

known. The presentation of HPV to the immune system in the epidermis and the local immune response is a topic of great interest, and information of diagnostic or prognostic significance may well be forthcoming from its study.

7. CONCLUSIONS AND FUTURE PROSPECTS

As may be seen from the previous Section, no one method is readily available at present for detecting genital HPV infections, although nucleic acid hybridisation, if well controlled, gives most information about the virus. From the point of view of the patient, assessments made at the time of colposcopic and other examinations are most valuable, especially if treatment is then immediate. A pathologist, working in conjunction with a clinician, can provide information rapidly using frozen sections of tissue. It remains to be seen whether the majority of HPV infections can be typed correctly on the distribution of nuclear atypia in sections. Much more requires to be discovered about the induction of dysplasia and what governs its progression, stationary phase or regression. Transmission of HPV types, especially of types 16 and 18, is important to ascertain and whether these viruses can be latent in the genital tract of men and women.

Nucleic acid hybridisation by Southern blotting of DNA from biopsies is a technique requiring expertise, and is expensive and time-consuming. However, recent advances using cervical scrapes in place of biopsies, probes labelled with biotin instead of a radioactive tag, and dot blotting have almost brought hybridisation into the realm of diagnostic virology. More information should be available in the near future from laboratories around the world about the association of HPV 6, 11, 16 and 18 with CIN, VIN and other neoplasms of the genital tract

which should give a clearer indication of their putative role in genital carcinomas.

While this book is devoted to the uses of monoclonal and polyclonal antibodies in diagnosis of sexually transmitted diseases, antibodies against HPV which are available at present do not provide all the information required. Antibodies which are type specific and/or which can detect early viral proteins are necessary and it should be feasible to produce such reagents. The only monoclonal antibodies used so far in HPV infections are ones which distinguish mononuclear cells in sections of tissues. This type of study is important, not so much in the context of detection of HPV, but in understanding the local immune response in lesions which progress, regress or remain stationary.

ACKNOWLEDGEMENTS

I wish to thank Dr S. Fletcher and Mrs H. Cubie for critical reading of the manuscript, and my colleagues for many helpful discussions. Dr S. Fletcher kindly provided Figure 8, Mrs H. Cubie Figures 1 and 4, Dr E. Pixley Figure 6, and Dr S. McNair Figure 7.

TABLE 1. Types of HPV and their predominant clinical lesions.

HPV Type	Lesion
1	Plantar wart
2	Common wart (mosaic type)
3	Plane wart
4	Palmar and plantar warts
5	Epidermodysplasia verruciformis (EV)
6	Condyloma acuminatum, flat condyloma, cervical intra epithelial neoplasia (CIN) grades 1 and 2
7	Butchers' hand wart
8	EV
9	EV
10	Plane wart, condyloma acuminatum
11	Condyloma acuminatum, flat condyloma, laryngeal papilloma, CIN 1 and 2
12	EV
13	Focal epithelial hyperplasia
14	EV
15	EV
16	Carcinoma of uterine cervix, vulva and penis, Bowenoid papulosis, vulval intra epithelial neoplasia grade 3, CIN 3
17	EV
18	As type 16

Table 2. The association of HPV with neoplastic changes in the uterine cervix

	% with koilocytes on cytology	% with koilocytes on histopathology		% HPV Antigen positive		DNA content % diploid	DNA content % aneuploid	HPV type by DNA hybridisation
CIN 1	80.6	79.6	95	28	43	33	8	HPV 6 or 11 42% HPV 16 or 18 18%
CIN 2	72	74	77	22	15	12	65	
CIN 3	58.1	62.8	54	9	15 in epithelium adjacent to lesion	0	82	HPV 6 or 11 8% HPV 16 or 18 50%
Cervical carcinoma								HPV 6 or 11 1% HPV 16 or 18 51%
	Baird (1985)	Baird (1985)	Kurman et al (1983)	Reid et al (1982)	Kurman et al (1983)	Reid et al (1984)		Reviewed in Gissmann (1984)

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F5

NUCLEAR DNA ANALYSIS OF KOILOCYTIC AND PRE-MALIGNANT LESIONS OF THE UTERINE CERVIX

Rhona G. Hughes, MBChB, William A. Neill, MPhil

and Mary Norval, PhD

Department of Bacteriology

University of Edinburgh Medical School

Teviot Place

Edinburgh EH8 9AG

(Telephone: 031-667-1011 ext 2210)

SUMMARY

Cervical biopsies were taken from 79 patients with various grades of cervical intra-epithelial neoplasia (CIN) or with koilocytic lesions of the uterine cervix, considered pathognomonic of human papilloma virus (HPV) infection, and from 10 women with normal cervices. The DNA content was analysed by flow cytometry. Analysis of data obtained showed that the biopsies from CIN and koilocytic lesions contained significantly more cells undergoing DNA synthesis and mitosis (29.89 - 33.06%) than those from normal cervices (21.60%). The proportion of aneuploid samples from the group with koilocytosis only and without CIN (18.2%) did not differ significantly from the group with CIN III (21.2%). These results have relevance for the postulated role of HPV in the aetiology of cervical carcinoma and for the clinical management of patients.

INTRODUCTION

The association between human papilloma virus (HPV) infection of the lower genital tract and intra-epithelial neoplasia has been recognised for some time and there is an increasing body of evidence to suggest that the association is causal rather than casual[1]. The lesions caused by papillomaviruses tend to be persistent and are characterised by increased proliferative activity. These features, as well as animal models[2] make the viruses attractive candidates for a possible role in human carcinogenesis.

Most human cervical carcinomas contain aneuploid cell populations[3] and a variable proportion of cases of cervical intra-epithelial neoplasia (CIN) are also aneuploid[4]. In addition it is known that the number of proliferating cells in a tumour increases with increasing malignancy[5]. Since the advent of flow cytometry it has become possible to identify aneuploid populations with greater accuracy and to quantify the fraction of a cell population in each phase of the cell cycle. In this study, we have used an EPICS flow cytometer to examine both these aspects in CIN and in HPV infection of the cervix.

MATERIALS AND METHODS

Patients

Seventy nine patients (age range 22-47) referred to the Elsie Inglis Colposcopy Clinic with abnormal cervical cytology or a clinically suspicious cervix were studied. Ten women undergoing minor gynaecological procedures (age range 25-43) with a normal smear history were included as normal controls.

Biopsies

A cervical biopsy was taken from an area of colposcopic abnormality within the transformation zone of each subject or from the transformation zone of the controls. The specimen was snap frozen on dry ice and stored at -80°C until processed further. A single 8 mm cryostat section was taken from the centre of each specimen and prepared for routine histopathology using a conventional haematoxylin and eosin stain.

The remainder of the biopsy was then thawed, minced and incubated in 3 ml 0.5% solution of pepsin (Sigma) in 0.01 M phosphate buffered saline (PBS), with the pH adjusted to 1.5 with HCl, at 37°C for one hour. The supernantant was drawn off and centrifuged at 2,000 g for 10 min and the resultant pellet washed in PBS and re-spun at 2,000 g for 10 min.

Staining procedure

The nuclear suspension thus obtained was stained using a modification of the method of Deitch et al[6]. The pellet was dispersed in 2 ml of a solution containing 50 mg/ml propidium iodide (Sigma) in 0.01M Tris (pH 7.0) with 5mM $MgCl_2$, 0.1% Triton X-100 and 15 µg/ml RNase (Sigma) and incubated at 4°C for 1-24

hours. The nuclei were then centrifuged at 2,000 g for 10 min and resuspended in PBS.

Controls for flow cytometry

Chicken red blood cells (CRBC) were run with each sample as an internal control. Their DNA content equals 35% of the DNA content of a human diploid cell[7]. They were stained using the propidium iodide solution described above.

Human peripheral blood lymphocytes were stained and run with CRBC as an external control at the start of each run.

Flow cytometry

The flow cytometer used was an EPICS V (Coulter) equipped with an argon ion laser, using 200 mW and emitting light at 488 nm. Nuclei were run at a rate of 150 per second until 20,000 stained nuceli had been counted.

Analysis of data

The data obtained were subjected to parametric analysis using the EPICS para 1 programme which determines the proportion of nuclei in each phase of the cell cycle. Statistical significance of each group compared to the control group, was calculated by the Student's 't' test.

RESULTS

Cell cycle analysis

Table 1 shows that of 89 cervical biopsies examined, 10 were classified by histopathology as normal squamous epithelium, 11 as containing areas of koilocytosis without evidence of CIN, 14 as CIN I, 21 as CIN II and 33 as CIN III. Koilocytosis was indicated by the presence of balloon-shaped cells in the epithelium and is considered pathognomonic of HPV infection[8]. Four specimens produced histograms on flow cytometry which, because of an abnormal distribution of DNA, were unsuitable for parametric analysis and they were omitted from this part of the study.

The table also indicates the percentage of cells in the S phase (the phase of DNA synthesis) and in the G_2 (the post synthetic gap) and M (mitotic) phases of the cell cycle. Fig.1 demonstrates the data obtained from one biopsy.

Traditionally, studies of cellular proliferation have examined the S phase fraction only, as it was not possible to quantify the cells in the G_2 + M phases using tritiated thymidine uptake. Two of us (RH and WN) analysed the histograms independently and found that the S + G_2 + M percentage was less subject to observer variation than was the S phase alone, although as can be seen from Table 1 the two measurements gave very similar results.

The S phase fraction rose from 11.97% of the total in normal epithelium to 20.64% in epithelium containing koilocytes but no CIN. This difference is significant ($p < 0.01$). The CIN

groups all contained significantly more S phase cells than the normal group. There was no significant difference between the koilocytosis and the CIN groups.

When the S and G_2 + M phases are considered together a similar pattern is seen. All 4 abnormal groups (Koilocytosis alone, CIN I,II and III) contain significantly more cells in the S + G_2 + M phases than the normal group but the 4 groups do not differ significantly from one another.

Aneuploidy

The DNA index was calculated from the ratio between the CRBC and sample modal channel numbers versus the ratio between CRBC and lymphocyte modal channel numbers. Thus a DNA index of 1 equals a diploid DNA content. A sample was considered to contain an aneuploid component if the DNA index was less than 0.9 or greater than 1.1 or if it contained a major subsidiary peak which was distinct from the G_0/G_1 (diploid) and G_2 + M peaks. All 4 abnormal groups included some aneuploid samples with the percentage ranging from 14.3% (CIN I) to 21.2% (CIN III). Of the 15 aneuploid samples identified, 6 were hypodiploid (DNA index < 0.9) and 9 were hyperdiploid (DNA index > 1.1). The DNA index of hyperdiploid populations was always less than 1.5.

DISCUSSION

We have shown that it is possible to distinguish normal cervical epithelium from epithelium containing koilocytosis and CIN using flow cytometry. Koilocytic epithelium and CIN differed from normal epithelium in containing a larger proportion of proliferating (S + G_2 + M phases) cells and, in some cases, possessing an aneuploid component.

It is particularly interesting to note that specimens containing koilocytes with no co-existent CIN differ so markedly from normal epithelium, both in proliferative activity and in containing some aneuploid populations (2/11 specimens). It has been postulated that papillomavirus infected cells initially behave as though stimulated by a non-specific mitogen and that, in a semi-permissive epithelium, many cells remain in a prolonged S or G_2 phase[9]. Reid et al[10] proposed that cumulative mitotic errors in these stem cells might result in occasional chromosomal mutations spawning clones of aneuploid basal cells. By demonstrating an increased proportion of S + G_2 + M phase cells in the koilocytic group our work would support this view. The CIN II and CIN III lesions were found to contain a smaller proportion of proliferating cells than the koilocytic and CIN I lesions (although the difference is not statistically significant). This fits Reid's hypothesis with the virus initially stimulating epithelial proliferation followed by subsequent S phase arrest and emergence of aneuploid populations.

In contrast to the work done by other groups[4,11] the relative frequency of aneuploidy did not increase significantly

with increasing severity of CIN in this study. The proportion of samples containing aneuploid populations was essentially similar in the 4 abnormal groups including the "koilocytosis alone" group (range 14.4% - 21.2%). To our knowledge this is the first published report of aneuploidy in "benign" HPV infection of the cervix and lends support to the hypothesis that HPV does have a central role in the aetiology of cervical carcinoma.

In addition there may be far reaching implications for the management of patients with any evidence of genital HPV infection or with CIN I. In many centres these women are treated conservatively in the hope that the lesion will regress. It has been suggested that the clinical behaviour of lesions may be predicted on the basis of the ploidy level[12]. Our data would indicate, on the other hand, that so called benign HPV infections and CIN III share similar DNA patterns and thus, it could be argued, should be treated identically.

ACKNOWLEDGEMENTS

We are grateful to Drs G.E. Smart and S. Fletcher for enabling us to obtain biopsy material from patients and clinical information, and for helpful discussions, and to the staff of the Colposcopy Clinic, Elsie Inglis Hospital for their assistance.

We thank the Cancer Research Campaign for funding this project, and the Melville Trust for a Clinical Fellowship to R.G.H.

Correspondence should be addressed to R.G.H., Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

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TABLE 1.

Percentage of nuclei from cervical biopsies in various phases of the cell cycle, and number showing aneuploidy.

Histopathology	Number suitable for parametric analysis	% S phase ± S.D.	% (S + G_2 + M) phase ± S.D.	Number aneuploid
Normal	10/10	11.97 ± 4.00	21.60 ± 4.51	0 (0%)
Koilocytosis alone	10/11	20.64 ± 8.62 $p < 0.01$	31.20 ± 8.53 $p < 0.01$	2 (18.2%)
CIN I	14/14	23.25 ± 9.36 $p < 0.001$	33.06 ± 8.71 $p < 0.001$	2 (14.3%)
CIN II	20/21	19.80 ± 7.74 $p < 0.01$	29.89 ± 8.39 $p < 0.01$	4 (19.1%)
CIN III	31/33	18.27 ± 5.8 $p < 0.001$	31.76 ± 8.38 $p < 0.001$	7 (21.2%)

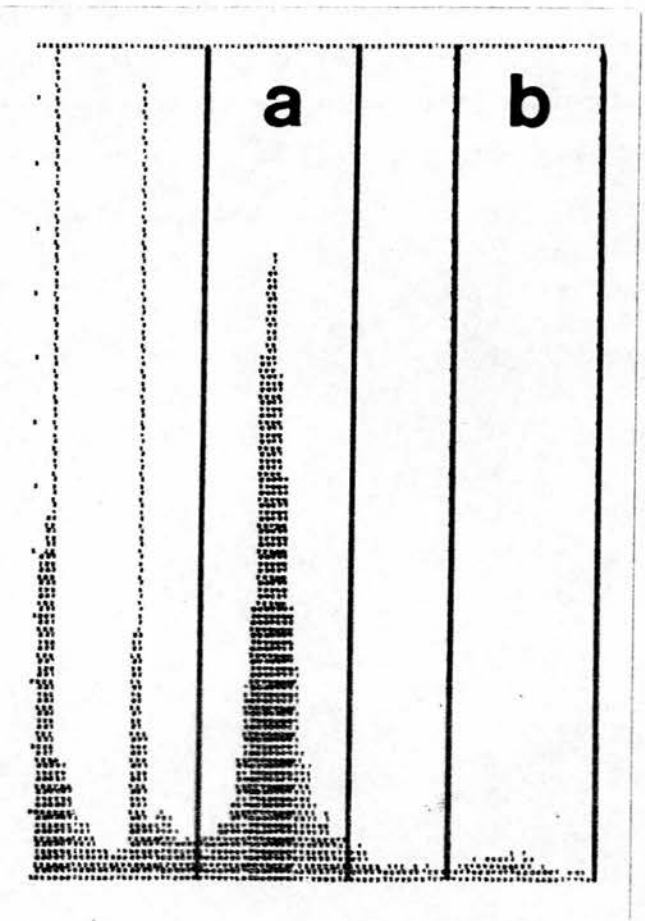
p values comparing each abnormal group with the normal group were calculated by Student's 't' test.

Legend for figure 1

Flow cytometry of nuclear DNA from a cervical biopsy

Red fluorescence is plotted on an arthrimetic scale along the ordinat and number of nuclei along the abscissa.

The G_0/G_1 peak lies within the first set of cursors (a) and the G_2 + peak set within the second set (b). The S phase fraction lies between the two sets of cursors and the chicken red blood nuclei from the peak to the left of (a).



MECHANISMS AND OUTCOMES OF PERSISTENT MICROBIAL INFECTIONS

by

Mary Norval, B.Sc., Ph.D.

A thesis presented for the degree of Doctor of Science,

University of Edinburgh, 1986

DEDICATION

To Tom

"Looking back at It

At nineteen I was a Brave Old Hunchback
Climbing to tremendous heights
Preparing to swing down on my golden rope
And rescue the Accused Innoncence.
But on my swooping, downward path one day
Innocence ducked
And, I amazed at such an act, crashed into
A wall she had been building,
How silly now to think myself able to rescue anything!"

DECLARATION

I declare that this thesis is my own composition. With the exception of papers A2 and A5 which describe work carried out as part of PhD studies (A1) at Edinburgh, no part of this thesis has previously been submitted towards a higher degree. In papers B1, D1, D2, D3, D10 and F1, I was a collaborator and not the senior author. In all the remaining papers, I was the senior author and main progenitor of the work described.

Mary Norval.

27/8/86.

المسمى الاسم .

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ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge the help and interest of my colleagues and friends. Dr Ian Sutherland, my PhD supervisor, sent me off on a voyage of discovery while I was an Honours student and, although I have changed direction considerably since those distant days, I still look back on the complex cell wall and extracellular products of bacteria with wonder. Professor Marmion influenced the change from my being a Bacteriologist to a Virologist and, in particular, initiated my interest in pathogenesis and in the factors involved in persistent viral infections. Dr Bill McBride, during numerous lengthy "chats", finally persuaded me that it might be worthwhile to consider some immunological mechanisms as being important in pathogenesis and his unfailing support through bad times and good is much appreciated. I must thank my veterinary colleagues for stimulating various interests and widening my horizons, namely Dr Mike Sharp, Dr Frank Scott, Dr Bill Martin, Dr Rod Else, Mr Ken Head and Miss Jean Robb. I am greatly indebted to my present group of colleagues, Dr Sarah Howie, Mrs Jean Maingay, Mrs Heather Cubie, Dr Jim Ross and Dr Rhona Hughes for their hard work, enthusiasm, good humour and for being such marvellously supportive people. Lastly to Bill Neill, the Virus Research Laboratory's senior and super technician over many years, a special thank you.

ABSTRACT

It is becoming increasingly clear that there are many viruses which are not eliminated from the body after the primary infection and which maintain a persistent presence thereafter, often with important consequences for the host. The exact mechanisms of these interactions are frequently uncertain. Most of this thesis is concerned with examining the association of various viruses with specific cells *in vitro* and *in vivo* which may lead to persistence, and ascertaining the outcome in terms of viral pathogenesis and immunity.

A viral aetiology for rheumatoid arthritis has been suggested for many years and several species, notably rubella, adenoviruses, measles and human retroviruses have suitable properties to make them putative candidates. Synovial material and mononuclear cells from patients with rheumatoid arthritis were examined for signs of virus in the form of infectious virions, viral antigens, viral nucleic acids, specific viral enzymes or a cell-mediated immune response, but no indication was found to substantiate a role for any of these viruses in the disease process. This work represents the only systematic and relatively wide ranging study on a viral involvement in rheumatoid arthritis.

Cytomegalovirus is known to persist in the body after the primary infection and it has been associated with several tumours in man, including adenocarcinoma of the colon and

cytomegalovirus in colonic and cervical tissue and an immune response to the virus did not substantiate this association. It was concluded that, while cytomegalovirus was occasionally detected in tumour material, it was not found sufficiently frequently to merit its suggested oncogenic role. In addition it was sometimes present in normal tissue, probably associated with T lymphocytes.

A study has been made of primary infection by herpes simplex virus type 1 (HSV) in a murine model. Irradiation of mice with sub-erythemal dose of ultra-violet B (UV) light led to a significant depression of delayed type hypersensitivity (DTH) response to HSV challenge. The suppression was mediated by two phenotypes of T suppressor cells. The ability of epidermal cells to present HSV antigens in an in vitro assay was also suppressed by UV-irradiation. The antigen presenting cells of the epidermis were capable of directing the type of immune response generated. These results indicate the importance of the initial interaction of the virus with the cells of the epidermis and how external events surrounding the infection may influence the immune response generated. There may also be consequences for the establishment of latency, and the frequency and extent of reactivation. A recrudescent model of HSV infection in the mouse has been developed. Urocanic acid present in the stratum corneum has been suggested as a possible photoreceptor and mediator of UV-induced suppression. Direct evidence was obtained that the cis-isomer of urocanic acid

induced suppression of DTH to HSV and that two subsets of T suppressor cells are involved.

Work has begun on examining the persistent nature of papilloma virus infections of the epidermis and the local immune response generated. Particular attention has been paid to human papilloma virus infections of the cervix, recently suggested as an important factor in the aetiology of cervical intra-epithelial neoplasia.

Studies have been made of various animal tumours, notably sheep pulmonary adenomatosis (jaagsiekte), canine and feline mammary carcinomas, and ovine adenocarcinoma of the small intestine, mostly by in vitro culture, examination of surface antigens and viral involvement, and tumourigenicity in athymic nude mice.

CONTENTS

INTRODUCTION

Most of this thesis concerns the mechanisms whereby viruses may cause persistent infections, how these agents accomplish a balance with the immune response of the host and what the outcome of such an interaction might be. However the initial research recorded here from 1969 was as a PhD student with Dr I.W. Sutherland, Department of Microbiology, University of Edinburgh, in the area of the biosynthesis of extracellular polysaccharides in bacteria. This work led to an interest in the control of complex polymer synthesis on bacterial cell surfaces and the implications of microbial products in pathogenesis. A short stay in the United States at the University of Wisconsin followed where the control of microcyst formation in Mycococcus xanthus, at the level of RNA polymerase, was examined.

A return was made in 1972 to Edinburgh and the Department of Bacteriology to join a research team led by Professor B.P. Marmion and the late Dr J. MacKay which was investigating whether any infectious agent might be involved in the aetiology of rheumatoid arthritis. At that time, the concept that viruses might be able to persist in the body after the initial acute or sub-acute infection was a novel one. Techniques were becoming available to monitor the "foot-prints" of viruses in cells without the need for an infection to be productive before it was apparent. In addition new information on Retroviruses had confirmed their unique method of replication, and oncogenes were

beginning to be discovered. The study, with Dr H. Hart and Mr W. Neill, of material from patients with rheumatoid arthritis was aimed at detecting viral antigens or nucleic acid in cells of the affected joints, or an abnormal cell-mediated immune response. In particular Retroviruses, rubella and measles, all good candidates as persistent agents in rheumatoid arthritis, were considered. A short sabbatical was spent in the laboratory of Dr C. Smith and Dr D. Hamerman, Montefiore Hospital, New York, in 1976. Although no evidence was obtained to associate any of these viruses with the initiation or continuation of the disease, important techniques were developed for the rapid and sensitive detection of viral information within clinical material. In addition mechanisms of persistence of viruses within a host were considered and possible outcomes of these interactions. During this time, some animal models and in vitro systems were described in the literature which showed a clear association of viruses with tumours.

With Dr Hart and Mr Neill, and later Dr A. Grail, all these factors led in 1978 to an examination of the association of cytomegalovirus, another persistent human virus, with cells of the immune system and a consideration of whether this virus might be involved in the initiation or progression of neoplasia. In particular cervical intra-epithelial neoplasia (CIN) and adenocarcinoma of the colon were studied using the methods developed during our rheumatoid arthritis project. No evidence was found to substantiate the suggestion that cytomegalovirus has

a role in these pre-malignant or malignant conditions.

A considerable interest was fostered in herpes virus infections during this work and in agents, other than cytomegalovirus, found in the cervix. In addition advances had been made during the previous few years on the concept of the skin acting as a major lymphoid organ of the body with its own antigen presenting cells and responding cells of the immune system. Thus a start was made in 1984 to look at persistent viral infections of the epidermis, in particular to quantitate local immune responses and their role in the progression, recurrence or resolution of the infection. With Dr S. Howie, Dr J. Ross and Mrs J. Maingay, a mouse model of herpes simplex virus infection was developed. Ultra-violet light and cis-urocanic acid were used as modulators of immune function. Human papilloma virus infections of the cervix were also examined, with Dr R. Hughes and Mrs H. Cubie, from the points of view of quantitating the local immune response and associating papilloma virus types with CIN. Part of this study was initiated while at the University of Western Australia in the laboratory of Dr J. Kulski and Dr E. Pixley.

Peripheral to these main lines of research have been studies with Mr K. Head of rumen papillomas in sheep, and with Dr R. Else of the behaviour of some canine and feline mammary carcinoma cell lines in vivo and in vitro. Finally, with Miss J. Robb, cell culture systems have been developed to screen for mycotoxins in complex extracts of animal feedings stuffs contaminated with

fungi and which may cause acute or non-acute symptoms in various animal species.

The following discussion outlines these areas in more detail and seeks to put the information obtained into the context of what was known at the time.

DISCUSSION

The publications which follow are divided into sections according to the area covered. Section A deals with work on exopolysaccharide biosynthesis in Klebsiella aerogenes. Section B discusses results obtained by examining tissues and cells from patients with rheumatoid arthritis for evidence of viral infection. Section C outlines studies on cytomegalovirus and its suggested role in adenocarcinoma of the cervix and carcinoma of the colon. Section D contains references on various animal tumours, sheep pulmonary adenomatosis, fibrosarcomas in mice, adenocarcinoma of the small intestine of sheep, feline and canine mammary carcinoma cell lines; also work on a cytotoxicity test for mycotoxins. Section E deals with herpes simplex virus infections in mice, and the effect of ultra-violet light and cis-urocanic acid on the immune response, and Section F on papilloma virus infections.

A final Section seeks to draw these areas together and to form some conclusions regarding the importance of persistent viral infections.

Section A BACTERIAL EXOPOLYSACCHARIDES

It has been known for a long time that bacteria produce polysaccharides as part of their cell walls and, in many species, polysaccharides are also synthesised extracellularly. Both tyes of macromolecules are important in pathogenesis and the control of their production is not well understood. In Klebsiella aerogenes the exopolysaccharide consists of neutral sugars and a limited range of uronic acids arranged in repeating units with, commonly, a single side chain. Several mutants were isolated which varied in their ability to synthesise exopolysaccharide from the parent strain (publications A1 and A2). One was unable to form discrete capsules and only synthesised exopolysaccharide as slime; the exact mutation was not determined and, even today, the mode of linkage of the capsule to the bacterial cell surface has not been elucidated. Another mutant was studied which showed reduced exopolysaccharide synthesis at low incubation temperatures and an effect on lipopolysaccharide synthesis leading to agglutination and "roughness" of colonies. The alteration was thought to be in an enzyme required to synthesise UDPglucose from glucose, or in an altered regulator, and it is still not known what controls the relative synthesis of lipopolysaccharide and exopolysaccharide. Indeed the distinction between the two polymers has become somewhat blurred as components thought to be confined to the lipopolysaccharide have been reported as integral units of exopolysaccharide.

It is important to determine the cultural and other conditions which favour exopolysaccharide synthesis and a study was made to determine the specific activity of enzymes involved in the synthesis of precursors in wild type and non-mucoid mutants of K. aerogenes grown under different conditions (A1 and A5). Little was known about the level of these enzymes or the control of precursor production, and, surprisingly, it was found that loss of ability to form exopolysaccharide or growth under various cultural conditions had little effect on the specific activity of enzymes tested; perhaps control then occurs at the metabolic level rather than at the genetic level. Sugar pyrophosphorylases may well represent one such point as they serve as the first stage commitment of the activated monosaccharides to polymer synthesis.

About 1968 it was known that the peptidoglycan backbone (Ghuysen, Tipper and Strominger, 1968) and the O antigen of lipopolysaccharide (Nikaido, 1968) were synthesised by adding monosaccharides sequentially from sugar nucleotide precursors to a lipid carrier, C_{55} isoprenoid alcohol phosphate, using membrane bound enzymes. The same information was not available for the synthesis of exopolysaccharides although it had been shown that a lipid carrier stage was probably involved. Our study (A3 and A4) showed that membrane preparations of K. aerogenes in a cell-free system were able to transfer sugars (glucose and galactose) from UDP derivatives to a lipid-soluble material and to a polysaccharide-resembling material. A model for capsular

polysaccharide synthesis was proposed whereby UDPglucose passes through the cell membrane and is modified to form the other sugar nucleotides which are then transferred sequentially to a lipid carrier before polymerisation and extrusion into the surrounding environment. The central role of the lipid carrier in bacterial polymer synthesis was considered. Since then cell-free synthesis of exopolysaccharide using lipid-free membrane systems and regenerated membrane has been accomplished (Troy, Frerman and Heath, 1971), and now cells made permeable to sugar nucleotides by EDTA are being used. This leaves enzymes in an ordered state so that sugars can be added sequentially. However much remains to be answered, such as the control of the molecular weight of exopolysaccharides and lipopolysaccharides, what controls the relative synthesis of these polymers and how they get transported through the bacterial cell membranes and walls.

In the past few years, there has been an upsurge of interest in exopolysaccharides as some are now important compounds in industry, the bacterial alginates and xanthan for example, while the complex interaction between the surfaces of bacteria and tissues of higher organisms in health and disease remain a largely undiscovered and important area.

Section B VIRUSES AND RHEUMATOID ARTHRITIS

It has been suspected for a long time that an infectious agent may be involved in the aetiology of rheumatoid arthritis and for the past twenty years strenuous efforts have been made to implicate specific viruses, especially those known to cause persistent infections in man. Our initial research sought to examine the cytology of synovial cells derived from patients with rheumatoid arthritis and to establish cultural conditions for their growth in vitro (Mackay et al, 1974; B1). Several cell lines were found which had the properties of transformed cells and which were thought to have arisen by co-cultivation of human Chang liver cells and synovial cells. No virus or viral products were detected despite using a variety of techniques available at that time. These included electron microscopy, induction followed by ^{3}H-uridine labelling, RNA and DNA polymerase activities and assays of viral antigens (B4). Methods have improved considerably since then and it would probably be worthwhile to re-examine these cell lines for, say, specific retroviral antigens, for viral nucleic acid and for abnormal oncogene expression. One cell line and synovial material from patients with rheumatoid arthritis were injected into the synovial cavity of baboons of different ages but they did not develop any arthritic joint symptoms despite being kept for three years (Mackay et al, 1983).

Retroviruses became attractive candidates in the aetiology

of rheumatoid arthritis after their unique method of replication had been described by Temin and Mizutani (1970) and by Baltimore (1970), and the realisation that the viruses could persist as integrated proviruses. For the next few years, there was some confusion about the properties of viral reverse transcriptase and how to distinguish it from a DNA-dependent DNA polymerase found in normal cells. Careful assays were performed directly on synovial material from patients with rheumatoid arthritis or on synovial cells grown in vitro from clinical material (B2 and B3). Synthetic template/primers were used and actinomycin D was present. No viral reverse transcriptase was found.

This approach was probably the only one available at the time but an attempt was made later to use nucleic acid hybridisation methods (B5 and B9). As techniques of cloning viral nucleic acids had not yet been described, four viruses, SV-40, rubella, a baboon retroviruses RD-114 and measles, which were thought to have suitable properties to make them possible agents in rheumatoid arthritis, were cultured in vitro. They were purified and their nucleic acid labelled with ^{125}I. This was used in membrane hybridisation with DNA and RNA of rheumatoid synovial cells and in in situ hybridisation but no positive cells were obtained. With improvements in the preparation and labelling of viral nucleic acids and in hybridisation techniques, the equivalent method today would be more sensitive and would have the potential to pick out sub-genomic quantities of viral nucleic acid or only a few cells in a population with viral information.

As yet, no one has reported such results using rheumatoid material although there have been many such publications from other areas, especially tumours.

It was suggested that the primary change in rheumatoid arthritis is in the effector cells of the immune system and that the rheumatoid process involves a loss of the normal immuno-regulatory control rather than a normal immune reaction against an altered target cell, such as a virus-infected fibroblast (Marmion, 1978). To test this hypothesis, synovial lymphocytes and monocyte-derived macrophages were examined for various viral antigens and viruses, retroviruses in particular. None were found (B7 and B8). Again no other workers have attempted this type of study and the series of experiments described above represent the only systematic study in the literature searching for a variety of viruses in a variety of cells from patients with rheumatoid arthritis.

In a review written in 1984, consideration was given to possible interactions between viruses and the host in rheumatoid arthritis (B10). Thus, while selected viruses remain as putative agents in the aetiology of rheumatoid arthritis, no definitive evidence has been obtained to date. It is crucial to examine material from early rheumatoid cases if an infectious agent is suspected, and to repeat some of the work reported here with more sensitive methods and including such viruses as the new family of human retroviruses. Effects on oncogene expression and better definition of any alteration in antigens on various

synovial cells leading to novel interactions with cells of the immune system would also make worthwhile studies.

During the course of this work, an interest was developed in a cell line persistently infected with rubella virus (B6). At that time there was some evidence to indicate that proviral DNA may be important in persistent infections involving RNA viruses. In our system, however, formation of defective interfering particles was found to be the major factor, not a DNA intermediate. Since then, apart from retroviruses, no RNA viruses have been described which persist as proviruses and the major mechanisms of persistence in vitro are defective interfering particles described here, development of temperature sensitive mutants and the protective effect of interferons. Which of these is important in vivo, if any, remains unclear and the interactions of the virus, infected cells and immune cells of the host are of importance in the pathogenesis of many persistent viral infections. Two such systems involving cytomegalovirus (CMV) and herpes simplex virus (HSV) are outlined below in Sections C and E respectively.

Section C CYTOMEGALOVIRUS AND ONCOGENESIS

CMV is a common human pathogen, frequently infecting children without producing clinical symptoms. Thereafter it persists for life, reactivating on immunosuppression and at other times. It is not clear where or how the virus persists. There is some evidence for it being involved in some specific tumours, namely Kaposi's sarcoma, adenocarcinoma of the colon and carcinoma of the prostate and cervix.

Our initial study was undertaken to find the range of interactions of CMV with various cell types. In mink lung epithelioid cells, infection was initiated but aborted at an early stage; rabbit lung fibroblasts allowed a low level of replication persisting over a long time (C1). A significant proportion of cells contained viral antigens even four weeks after infection but extracellular virus was not demonstrable. CMV may have become latent in some rabbit cells with only a few cells being capable of producing infectious virus. Interferon may be an important factor in this persistence but whether such a model may be applied to any human cell type is not known.

The association of CMV with human mononuclear cells is of interest and evidence was obtained to indicate that the virus interacted specifically with T cells in vivo and in vitro (unpublished). Peripheral blood monocytes, alveolar macrophages and peritoneal macrophages were all resistant to infection. The T cells may represent a possible site of latency of CMV and, if

so, the virus-T cell balance may be crucial in reactivation and in the regulation of the general immune response.

The role of CMV in cervical intra-epithelial neoplasia (CIN) was examined using several approaches. Serological surveys in the literature had given equivocal results about the levels of antibodies to CMV in patients with CIN, depending often on the assay used. Each serum was tested by three methods which should detect antibodies to different CMV antigens and no correlation was found between antibody titre and pathological diagnosis (C3). Peripheral to this study was the finding that the frequency of carcinoma in situ was not age-dependent, thus emphasising that a national screening programme should include all sexually active women. This policy is gradually being adopted.

In addition to CMV antibodies, zinc and copper levels were measured as an indicator of the presence of a malignant tumour and its stage; and low levels of zinc may reflect low levels of vitamin A which had been correlated with the development of CIN. It was concluded that zinc and copper levels could not be used as a diagnostic aid to CIN and little support was obtained for the proposal that low serum zinc levels are associated with CIN except, perhaps, at the malignant stage of invasive carcinoma (C7).

Peripheral blood lymphocytes from CIN patients were assayed for responsiveness to CMV antigens using a lymphocyte stimulation test but there was no difference between patients with various degrees of CIN and controls (C6). In addition natural killer

and killer cell activity of CIN patients did not differ from controls, and there was no correlation between antibodies to CMV and increased natural killer cell activity (C4). Thus no evidence was found to support the view that patients with cervical cancer had reduced natural killer cell activity as had been reported previously (Pulay, Benczur and Varga, 1982).

No CMV mRNA was found in CIN biopsies by *in situ* hybridization and no viral antigens by immunofluorescence. Cultured endocervical cells were permissive for CMV if in close contact with connective tissue fibroblasts (unpublished).

Other studies were done to clarify the role of CMV in adenocarcinoma of the colon. No viral nucleic acid or antigens were detected in colonic biopsies or after culture of biopsies *in vitro* (C2). This was not in agreement with published results (Huang and Roche, 1978; Hashio, Horikami and Loh, 1979) but since then other groups have confirmed our conclusions (Brichacek et al, 1980; Roche et al, 1981). It was thought possible that antibodies could be present on colonic cells, thereby blocking both antigenic sites and receptors and halting viral replication. However, eluates from adenocarcinomatous tissues showed the same level of antibodies to CMV as eluates from normal colonic tissues (C5). A final publication in this area outlines the effect of molecular binding to the cell surface on the regulation of intra-cellular events during CMV replication in permissive cells (C8). It was known that CMV induced Fc receptors on infected cells which probably share some identity with the receptors for

antibodies and concanavalin A or succinyl concanavalin A due to a reduction in cell viability. This may have some significance for persistence of CMV in sites such as the colon or cervix.

Our studies led to the conclusion that CMV does not play a significant role in either of the two human malignancies examined. Unlike most other groups, the approaches used covered a wide range, from detection of viral nucleic acids and antigens to serological and cell-mediated immune responses. It has been proposed that some herpes viruses may act as mutagens by a "hit and run" mechanism (Galloway and McDougall, 1983) and this type of association of CMV with tumours, especially if the event happened early in the tumour induction, cannot be excluded by our findings. However, some specific immune response might still be expected but this was not found. CMV was detected very infrequently in colonic and cervical tumours during the course of these studies but was present, on occasion, in normal tissues. Thus CMV may persist in sites such as the cervix and colon, perhaps associated with T lymphocytes, but not specifically in pre-malignant or malignant tissues.

Section D VETERINARY-RELATED STUDIES

Collaborative studies resulted in publications D1, D2 and D3. For the first time, in 1976, sheep pulmonary adenomatosis was produced in experimental sheep using a putative retrovirus alone or, more efficiently, in conjunction with an ovine herpesvirus, raising the possibility of synergism between two viral agents (D1). The methods of purification of the retrovirus relied on techniques developed in the rheumatoid arthritis project already outlined in Section B, and the presence of the virus was assumed from its reverse transcriptase activity. Further work has since confirmed the presence of the retrovirus and characterised the reverse transcriptase enzyme (Herring et al, 1983). A collaborative project is currently underway to clone part of the viral genome coding for the major core protein and to prepare monoclonal antibodies to this protein.

A very interesting and, at the time, unique study demonstrated that normal human tissues contain nucleic sequences related to Adenovirus type 2 and expressed as mRNA (D3). Using reagents obtained from the rheumatoid arthritis project, it was shown that these RNAs were translated into proteins which may be important in some tissues during cell growth and differentiation. Since then, sequences, often G-C rich, of other viruses, such as herpes simplex virus and CMV have been reported as normal human tissue. If found in tumours, misleading conclusions have sometimes been formed concerning the role of viruses in the

aetiology of the tumour. Presumably these regions are similar in sequence to selected sequences in human DNA and perhaps the protein products of such regions are also similar allowing cross reaction in immunological tests. The whole area is one of immense interest at the present time with advances in understanding of oncogenes, integration of viral genes and mechanisms of cell growth.

Emphasis was placed in D2 on making sure that cells grown in vitro and then used for in vivo tumourigenesis were free of contamination with viruses or Mycoplasma. Such tests remain important and are rarely undertaken.

An interest in tumour cells led to an in vitro study of adenocarcinoma cells from the small intestine of sheep (D4). Morphology, ultra-structure and any involvement of viruses were noted. One cell line was obtained which was examined in detail as a mixture of epithelial and other cell types were present throughout many subcultures, thus mimicking the appearance of the tumour in vivo. The epithelioid cells were separated from the others and their properties indicated that they represented carcinoma cells. No other ovine cell line from any tissue has been reported since then. These results and a possible viral aetiology in adenocarcinoma of the small intestine prompted a subsequent investigation of ovine rumen papillomas reported below in Section F.

Canine mammary carcinomas were examined by in vitro culture and three epithelial cell lines were obtained which induced

tumours in athymic nude mice (D5, D6 and D7). These lines are the only canine ones reported and lately an equivalent feline mammary carcinoma line has also been established (D9). The interest in such lines lay in examining their surface properties in vitro such as oestrogen receptors and in establishing a nude mouse model of tumour production, in particular for experimental hormone manipulation as an alternative to using dogs. There is a high incidence in bitches of naturally occurring mammary carcinomas which are similar to human tumours in their pathology and receptor status, and thus this work was also of value in the medical field. Tamoxifen has been used in the treatment of breast cancer in women for several years and is thought to act at least partly as an oestrogen antagonist, although, in some surveys, no correlation was found between oestrogen receptor status and response to tamoxifen treatment. One canine mammary carcinoma cell line, REM-134, had no detectable oestrogen receptors and induced tumours at the same rate of growth in male and female nude mice. The effect of tamoxifen on the tumours induced by REM-134 cells was ascertained (D8). Oral tamoxifen reduced the rate of growth of tumours and to the same extent in male and female mice. These results argue against tamoxifen acting solely as an antagonist for oestrogen and in vitro studies support this finding. Two trials are currently underway to test the action of tamoxifen in tumour-bearing bitches and it will be interesting to find out whether the nude mouse model is relevant to the situation in dogs (Else and Pierrepoint, personal

communication).

Finally in this Section, a collaboration was established as a result of an episode of disease in broilers in 1980-81 in Scotland (D10). This was toxin-related and a large number of fungi of Fusarium species were isolated from mould-contaminated maize and wheat components of the feedstuff. In the past few years, it has been recognised that mycotoxins can be important factors in causing episodes of sub-acute disease or occasionally of acute disease in a variety of animal species. While some of these mycotoxins such as aflatoxin or zearalenone have been identified and well-studied, others remain totally uncharacterised. The normal method of identification of mycotoxins was by chromatography after solvent extraction of fungal mycelium or contaminated material, and to compare the result with standards of known and purified mycotoxins. A screening test was thus required which would detect unknown as well as known toxic fungal metabolites. The sensitivity of various cell lines to several purified mycotoxins was established (D11 and D12) and it was concluded that such a cytotoxicity test was a useful addition to chromatography, being significantly more sensitive for the trichothecenes in particular. It is of special use if a variety of cell lines with different receptors and different enzymes are employed, such as Chang liver cells for hepatotoxins or Neuroblastoma cells for neurotoxins. Currently an examination is being made of hydroponic barley associated with a fatal haemorrhagic syndrome in cattle, and of malt culms associated

with fatal neurological symptoms in sheep. The cytotoxicity test is now employed in many laboratories screening for mycotoxins such as the Ministry of Agriculture. It is of particular importance as a precautionary assay in cases where diet and weight gain are tightly controlled and where feed is given from bulk supplies as is happening more frequently in present day agricultural practices. It is also useful in episodes of unexplained illness, associated with mouldy feeding stuff.

Section E HERPES SIMPLEX VIRUS INFECTIONS

Primary childhood infections with herpes simplex virus (HSV) are common and, in at least 50% of cases, the virus becomes latent, being reactivated at intervals throughout life. Although much is known about the replication of the virus in permissive human cells and about the humoral and cell-mediated immune response generated by the infection, little work has been done to examine the interaction between HSV and antigen presenting cells (APC). These cells are recognised to be of primary importance in generating the immune response and in resistance to viral infections.

In the first place (E1 and E2) murine bone marrow cells were cultured in vitro and underwent maturation and proliferation in the presence of L-cell conditioned medium. When infected with HSV, differentiated cells exhibited a cytopathic effect although no infectious virus was produced and most were resistant to infection. However the Major Histocompatibility (MHC) Class II antigen expression in the culture was increased by the infection, perhaps due to interferon production. This result may have important implications for the generation of the local immune response to HSV where the number or efficiency of potential APC at a particular site may be increased by increasing the MHC Class II antigen expression. Few groups have looked at the association of viruses with homogeneous macrophage populations and it would be of particular interest to examine purified

Langerhans cells, the major APC of the skin, in this way. This project is currently underway using fluorescent cytometry and anti-Class II monoclonal antibodies.

Secondly a model of HSV infection was developed in C3H mice to examine cell-mediated immune responses, in particular delayed type hypersensitivity (DTH) which is known to be important in viral clearance from a local site. It had been shown before that irradiation with ultra-violet (UV) B light suppressed DTH to contact sensitising agents (Noonan et al, 1984) and to tumour anitgens (Fisher and Kripké, 1978). An attempt was therefore made to show the same effect with viral antigens. If the mice were pre-irradiated with a sub-erythemal dose of UV-B between 2 and 14 days before being infected with HSV, then their DTH response was suppressed by 60-90% (E3). The nature of the suppression was tested by transfer of spleen cells from UV-irradiated mice to mice previously infected by virus. Suppression of DTH resulted and was shown to be due to T suppressor cells in the transferred spleen population which were of two phenotypically distinct subsets, $Lyl^{+}2^{-}$ and $Lyl^{-}2^{+}$ (E5). The ability of epidermal cells to present HSV antigen in an <u>in vitro</u> assay was decreased by at least 60% if the mice were UV-irradiated before preparing the epidermal cells (E4). It has also been shown that epidermal cells from mice pre-irradiated with UV induce suppression to HSV in naive mice if injected in the same site as the virus (E8). In this case only one type of T suppressor cell was generated and the APC were thus directing

the type of immune response. This is a new and exciting concept that the state of the APC on first encountering the antigen can be a major factor in the type of immune response generated.

All these results have profound significance for the nature of the virus-host relationship. UV-irradiation in a critical period prior to contact with HSV may determine the extent and severity of the primary infection, which infections become latent and which recrudesce and with what frequency and severity. The effect of UV-irradiation on viral infections of the skin has been reviewed in E6.

While UV-irradiation is known to cause local and systemic effects, its mechanism of action is not clear. Urocanic acid, found preferentially in the stratum corneum, has been suggested as a possible photoreceptor mediator (De Fabo and Noonan, 1983), converting from the trans to cis form on irradiation. Using the murine model already outlined, cis-urocanic acid applied epidermally or injected subcutaneously before sensitisation with HSV led to the suppression of DTH response on subsequent challenge with HSV (E7). This was the first direct evidence that urocanic acid can induce suppression to an epidermal antigen. The suppression was due to the induction of specific T suppressor cells of two phenotypic subsets, likely to be the same as those induced by UV-irradiation (E9). It is now planned to ascertain the site and mode of action of urocanic acid.

In addition to primary HSV infections, attempts have been made to adapt the murine model to examine recrudescent

infections. Recently this has proved successful (manuscript in preparation). Mice were UV-irradiated before being infected epidermally with HSV to give visible lesions. Thus their initial immune response was somewhat suppressed and altered by irradiation. After two months, they were irradiated again, and tape stripped at the site of the initial lesion. In over 80% of mice, lesions appeared within two days either at this site or at an adjacent site. This finding allows an examination of the immune response surrounding recrudescence; in particular, the role of contra-suppressor cells in latency can be tested.

Thirdly, recrudescent oral HSV infections in man are being studied. Mononuclear cells from peripheral blood have been used in a lymphoproliferative test with HSV as antigen. The blood cells have been depleted of APC by glass adherence and the role of Class II MHC antigens in antigen presentation found by blocking studies using monoclonal antibodies to the various regions of the Class II MHC locus. Epidermal cells from suction blisters are being tried as APC in the lymphoproliferative test. It is planned to include patients with eczema herpeticum in this study. Preliminary results indicate a poor lymphoproliferative response to HSV antigens at the time of clinical lesions with generation of natural killer cells.

The studies outlined above represent a concerted effort to understand the events surrounding the initial interaction of the virus with the cells of the host. They are unique in that a clinical isolate of HSV was used throughout and most experiments

have involved epidermal infections. The epidermal route was chosen deliberately as being more natural than subcutaneous, intravenous or intra-peritoneal which are commonly used by other investigators. The dose of UVB irradiation necessary to effect the immunosuppression was sub-erythemal and raised questions about the frequency of transient immunosuppression after exposure to sunlight and the consequences for persistent viruses already present in the body or, indeed, for any microbial pathogen encountered for the first time during the immunosuppression.

Antigen presentation is of fundamental importance to the generation of the immune response and it is significant to find that the APC may be altered by external circumstances to induce suppression instead of a positive immune response. The results with cis-urocanic acid represent the first time that this compound has been shown to regulate the immune response directly, and considerable efforts are being made to pinpoint its site and mechanism of action. If it is possible to transiently immunosuppress by painting the skin with cis-urocanic acid, then such a procedure may be of clinical benefit in skin grafting. Finally development of a murine model of recrudescent HSV infections leads to the possibility of the analysis of the local immune response surrounding reactivation by the same techniques as in primary infections. Contra-suppressor cells have been implicated in maintaining local immunity while systemic hypersensitivity is prevented. During the triggering period of reactivation, this balance may be lost leading to a local

suppression of immunity and allowing virus to replicate in epidermal cells. This hypothesis is currently being examined.

Section F PAPILLOMA VIRUSES

As already outlined in Section C, several viruses have been associated with cervical intra-epithelial neoplasia (CIN), the latest and most convincing of which is human papilloma virus (HPV). It is now recognised that a high proportion of patients with CIN have evidence of infection with specific HPV types (Gissmann, 1984). In the superficial layers of the epithelium, HPV infection is thought to be productive with formation of characteristic koilocytes although in the cervix, mature virus particles are rarely detected and never in large numbers. In a hypothesis in 1983 (F1) the possible association of HPV with the deep cells of the squamous cervical epithelium was considered. If there is dysplasia in the deep layers, HPV could be non-causative or causative of this state. In the former case, the association between the virus and CIN is unrelated but, perhaps, the virus becomes activated or is able to infect cells transformed by some other means. In the latter case, the virus is associating with the deep cells to cause dysplasia. Thus the virus and epithelial cells are interacting in different ways depending on the degree of differentiation of the epithelium, and perhaps depending on other factors such as co-infection with another virus or synergism with a carcinogen. If the deep cells are not dysplastic, perhaps this represents an early non-koilocytic or pre-koilocytic stage leading to death of maturing cells and a return to normal epithelial appearances.

This hypothesis remains to be fully tested and is of obvious importance in resolving the causal or casual role of HPV in genital cancers. With advances in *in situ* hybridization techniques, and *in vitro* culture methods for cervical biopsies and in analysing the function of regions of the HPV genome, these interactions should become clearer.

Recently it has become possible to analyse nuclear DNA from human material using flow cytometry. Phases of the cell cycle and aneuploidy can be calculated. A survey was undertaken to analyse cervical biopsies from patients with CIN and with histopathological evidence of HPV infection without CIN (F5). Evidence was obtained to show that HPV induced cellular proliferation and to approximately the same extent as found in CIN grades. The percentage of samples which were aneuploid was the same in each CIN grade and in the HPV infection without CIN group. Thus it appears that HPV infection induces abnormal cellular proliferation and changes in the DNA content in a certain proportion of cases. These results have significance for the management of women with cervical HPV infections without CIN or with CIN I who are often left untreated in the hope that the lesion may spontaneously regress. This may not be a wise policy if aneuploidy is already present and if the epidermal cells are proliferating abnormally.

Interest in genital HPV infections has grown rapidly over the past few years and it has become desirable not only to detect HPV infection but also to type the virus. A chapter was

recently written on this topic (F4) outlining various methods available including nucleic acid hybridization with labelled HPV DNA probes under different stringency conditions. Our particular interest is in the local immune response to HPV infections, especially the role of antigen presenting cells in persistence, regression or progression of the lesion.

On a wider front, nucleic acid probes are beginning to be used for rapid viral diagnosis and offer some advances over more conventional methods. This topic was reviewed in F2.

Finally in this Section, reference is made to work done on rumen papillomas in sheep (F3). It arose out of the study described in D4 on adenocarcinoma of the small intestine in sheep as it seemed possible that there may be a link between the papillomas and the development of the malignancy. This has been described for alimentary papillomas and carcinomas in cattle, with bracken fern in the diet being an important co-factor (Jarrett et al, 1983). It had been noted that sheep grazing on local moorland sometimes had rumen papillomas occurring in conjunction with adenocarcinoma of the small intestine. The incidence of rumen papillomas was 12.5% in sheep slaughtered in Edinburgh - a surprisingly high figure, especially as ovine cutaneous papillomas are extremely rare. Although evidence was obtained to indicate the infectious nature of the papillomas, neither virus particles nor viral DNA were found and only a few cells in the uppermost epithelial layer were expressing viral antigens. This result is consistent with any virus producing

cell being shed rapidly from the surface of the mucous membrane in the rumen. There may be a similar situation in the human uterine cervix. It would be necessary to clone an ovine papilloma virus DNA and to use this as a probe to screen both rumen papillomas and intestinal adenocarcinomas for viral information. Unlike this country, cutaneous papillomas on unwoolled areas of sheep are reported frequently in Australia and a collaborative survey is underway with the University of Western Australia to examine their possible conversion to carcinomas, with UV-irradiation as a suggested co-factor.

IN CONCLUSION

The preceeding Sections seek to discuss published and unpublished work of the past sixteen years. It has been of interest to see how rapidly some techniques have developed during this time, notably in detecting viral components in infected cells. Yet there remain many questions to be answered concerning persistent infections. It is no longer adequate to study viral infections of specific cells without considering the implications for the immune response of the host; conversely, the host may play just as important a role as the virus in creating conditions for the persistence. What seems certain from the various viruses studied here is that different mechanisms of interaction may pertain in each case. With regard to tumours, a multistep induction is most likely with viruses, perhaps, playing some role in one or more of these stages. Other factors are probably as important as viral infection like chemical carcinogens, co-infections, genetic background, age and state of differentiation of tissues. The persistent nature of most viruses associated with tumours is recognised but this does not rule out a "hit and run" mechanism or a specific chromosomal translocation event directed by an integrated viral genome. It is impossible, as yet, to detect small regions of integrated viral nucleic acid or rearranged sequences and the interaction of viral genes with cellular oncogenes remains to be evaluated in most systems.

A study of the effect of viruses on the immune response, either direct or indirect, is important. The primary event in the immune response occurs when the antigen is processed by the antigen presenting cells which then present fragments of the antigen in association with Class II MHC molecules on their surfaces to T cells. Much remains to be found out about the actual process of antigen presentation and the effect on the generation of specific lymphocyte subclasses. At the present time, as outlined in Section E, we are examining epidermal viral infection and the role of Langherans cells, the major antigen presenting cells of the skin. The association of viruses with this cell type is largely unknown and how various environmental factors may affect this interaction is being studied. Questions remain to be answered about the individual's response to a virus, what determines establishment of latency, what affects frequency and extent of reactivation and if there is any association with neoplasms or immune complex disease.

It is becoming clear that it is not unusual for many virus species to persist in the body after the primary infection and that they may re-appear in different guises or perhaps affect the immune system in more subtle and, as yet, poorly defined ways. The interface between viral pathogenesis and immunity in persistent infections is one of interest and fascination at the present time, not only to virologists and immunologists but also to oncologists, molecular biologists, cell biologists, epidemiologists and clinicians.

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J. gen. Microbiol. (1969), **57**, 369–377
Printed in Great Britain

A Group of Klebsiella Mutants Showing Temperature-dependent Polysaccharide Synthesis

By MARY NORVAL AND I. W. SUTHERLAND
Department of General Microbiology, University of Edinburgh College of Agriculture, West Mains Road, Edinburgh EH9 3JG

(*Accepted for publication 25 April* 1969)

SUMMARY

Several mutants isolated from three *Klebsiella aerogenes* strains showed characteristics differentiating them from the wild type and also from the normal type of non-mucoid mutants. These mutants synthesize much less polysaccharide slime or capsule than the parent bacteria at low incubation temperature, but similar amounts at 37°. The colonies are of rough appearance at 20° and liquid cultures at this temperature autoagglutinate. At 37°, mutant and parental types are indistinguishable. The mutants show altered phage sensitivity patterns at 20° and the yield of lipopolysaccharide appears to be decreased at lower temperatures. Double mutants retaining certain of these characteristics but devoid of exopolysaccharide-synthesizing activity have also been isolated. The properties of the parent and mutant types are compared.

INTRODUCTION

In studies on polysaccharide biosynthesis, frequent use has been made of mutants unable to synthesize the exopolysaccharide slime or capsule characteristic of the parent organism, for example, *Diplococcus pneumoniae* capsular polysaccharide (Smith, Mills & Harper, 1957), the slime polysaccharide synthesized by *Escherichia* strains and by other species of the Enterobacteriaceae (Beiser & Davis, 1957). During studies on exopolysaccharide biosynthesis by strains of *Klebsiella aerogenes* a number of mutants differing from normal non-mucoid variants were isolated. The unusual properties of these mutants and their relationship to the parent strains are reported.

METHODS

Bacteria and methods of culture. Three strains of *Klebsiella aerogenes* have been used. Strains A3(SL) and A4 are laboratory strains used in several earlier studies (Wilkinson, Dudman & Aspinall, 1955; Sutherland, 1967). Strain W70 was kindly provided by Dr D. G. McPhee, School of Biological Sciences, University of East Anglia. Strains KP1 and A4-O are non-mucoid mutants of A3(SL) and A4, respectively, and are included in certain experiments for comparison. All strains were cultured routinely on nutrient agar at 35°. For production of extracellular polysaccharide, strains were grown on a solid nitrogen-deficient medium (Sutherland & Wilkinson, 1965). A liquid version of this medium was also used for some experiments. The minimal medium used was that described by Davis & Mingioli (1950). Liquid cultures were grown in Erlenmeyer flasks containing half the nominal volume and shaken at 200–300 rev./min. in an orbital incubator. Initial isolation of mutants was made on

eosin methylene blue (EMB) agar with glucose as carbon source or on nitrogen-deficient medium.

Bacteriophages. The phages having *Klebsiella aerogenes* A3 (SL) as host have been described earlier (Sutherland, 1967). Bacteriophage active against other *K. aerogenes* strains were isolated from raw sewage by the same techniques except that membrane filtration and not chloroform was used to remove bacteria from the preparations. The phage-induced fucosidase preparation was that used by Sutherland (1967).

Mutagenesis. 'Old cultures' were nutrient-broth tube cultures held at 35° for 10 to 20 days. Dilutions were made into sterile 0·9 % (w/v) saline and 0·1 ml. samples were spread over the surface of suitable solid media. 2-Aminopurine mutagenesis was performed on overnight nutrient broth cultures which were diluted to approximately 10^4 bacteria/ml. in broth containing 2-aminopurine (200 μg./ml.), and incubated 48 hr at 35°. Samples were then spread over nitrogen-deficient solid medium.

Preparation of polysaccharides and lipopolysaccharides. The exopolysaccharides produced by the three strains were of two types: slime or capsule. These were isolated and purified by the techniques originally described by Wilkinson *et al.* (1955). Lipopolysaccharides were extracted from lyophilized bacterial cells by the phenol+water method using a 5 min. extraction time (Lüderitz *et al.* 1965). After removal of the phenol by dialysis, the extracts were concentrated under reduced pressure and the lipopolysaccharide was freed from other material by ultracentrifugation at 100,000 *g* for 4 hr, then lyophilised. The supernatant fluid was also freeze-dried and assayed for glucose as a measure of glycogen.

Microanalysis. The constituent sugars of the polysacharides and lipopolysaccharides were identified by paper chromatography in the solvent of Fischer & Dörfel (1955), after hydrolysis in N-H_2SO_4. The individual sugars were determined as described previously (Sutherland, 1967). In addition, mannose was determined by the cysteine+sulphuric acid assay (Dische, Shettles & Osnos, 1949) and total polysaccharide by the anthrone method. The *O*-acetyl groups found in two of the three exopolysaccharides were estimated by the hydroxylamine-ferric chloride assay (Hestrin, 1949). Total protein was assayed by the Folin technique. All colorimetric results were read in glass cells in a Zeiss PMQII spectrophotometer or a Unicam SP500 instrument. These were also used for determining the turbidity of cell suspensions and for enzyme assays.

Paper chromatography and paper electrophoresis. Paper chromatograms on Whatman no. 1 paper were run in ethyl acetate+acetic acid+pyridine+water (5+1+5+3, by vol.) (Fischer & Dörfel, 1955) or ethyl acetate+acetic acid+formic acid+water (18+3+1+4, by vol.) (Feather & Whistler, 1962) for 24 or 40 hr respectively. Partial acid hydrolysates (N-H_2SO_4 for 15 min. at 100°.) were neutralized with $Ba(OH)_2$ solution and compared by paper electrophoresis in pyridinium acetate at pH 5·3 using the conditions previously described (Sutherland & Wilkinson, 1968), and by paper chromatography in the acid solvent.

RESULTS

Isolation of colonies with crenated colony type (CR mutants).

The parent strains were subjected to mutagenesis and spread, after expression of mutations, on nitrogen-deficient or EMB medium and the colonies examined for the presence of non-mucoid types (O mutants). These occurred at low frequency and were

slightly smaller than the wild-type colonies, lacking the mucoidness and consequent glossy appearance. The non-mucoid cells grew in liquid culture with uniform turbidity and had a growth rate similar to the parental type. Several hundred O mutants have been isolated using various mutagens. During examination of colonies following mutagenesis, another type of mutant was observed. These colonies (CR mutants) were detected on plates incubated at 30° or 20° and had a very characteristic appearance. They were of the same size as those of non-mucoid mutants (10 to 12 mm. diam. after 48 hr growth on nutrient agar at 20°). The outline of the colonies was irregular and the consistency more friable than either the parental or O type, and ridges radiated from a central apex. The CR mutants were only isolated following treatment with aminopurine and not with other mutagens. One CR mutant was obtained as a spontaneous mutant from an 'old culture'. From two of the CR mutants further mutagenesis with aminopurine led to the isolation of another form which we have designated CR-O mutants. These cultures possessed properties common to CR mutants and O mutants. The derivation of mutants and their type is shown in Table 1. The frequency of occurrence of either CR or CR-O mutants was very much lower than that of the O mutants when aminopurine was used as mutagen. Attempts to derive a CR-O mutant from *Klebsiella aerogenes* W70 have been unsuccessful.

Table 1. *The type and mode of isolation of the mutants used in the present study*

Parent	Mutant	Type	Mutagen
A3(SL)	A3-CR	CR	2-Aminopurine
A3-CR	A3-CRO	CR-O	2-Aminopurine
W70	W70-CR1	CR	2-Aminopurine
W70	W70-CR2	CR	2-Spontaneous
A4	A4-CR	CR	2-Aminopurine
A4-CR	A4-CRO	CR-O	2-Aminopurine

Cultural characteristics of CR mutants

The characteristic colonial appearance of CR mutants on solid media was only observed following incubation at 20 to 30° for 24 to 48 hr. After longer incubation at these temperatures some mucoidness was noticed. The colonies also become less friable and very adhesive to the medium. Incubation at 37° led to cultures almost indistinguishable from the wild-type strains. The CR-O mutants were recognizable as their characteristic colonial appearance was retained on incubation at 37°. They thus appeared to lack the ability to synthesise extracellular polysaccharide, so resembling O mutants. Colonies on solid media were very friable.

Liquid cultures of CR mutants at 20° in all the media tested underwent autoagglutination and sedimentation as a granular aggregate of bacteria. At 37° liquid cultures were indistinguishable from the wild type, being turbid and viscous due to polysaccharide excretion. The CR-O mutants showed the same autoagglutination and lack of polysaccharide production at all incubation temperatures. The autoagglutination of CR or CR-O mutants in liquid defined media was unaffected by altering the concentration of Ca^{2+} or Mg^{2+} ions between 0 and 0·5 mM and by the addition of EDTA up to 3 mg/ml. (w/v). Addition of Tween 80 in concentrations up to 1 % (w/v) also failed to prevent autoagglutination. All strains grew in the same manner using glucose, galactose, mannose or glycerol as carbon and energy source in defined media.

Reversion

A marked difference between CR and O mutants was in the stability of the mutation. In the *Klebsiella aerogenes* A4-O and other non-mucoid variants spontaneous revertants were never detected despite examination of more than 10,000 colonies. Application of various mutagens did not induce reversion. The same was true of the CR-O mutants. However the CR mutants reverted to the parental type with varying frequencies. Thus A3-CR reverted to A3(SL) spontaneously with 0·01 % frequency, whereas no spontaneous revertants from A4-CR were observed. The use of acriflavin, 2-aminopurine or ethanemethanesulphonate resulted in a reversion frequency of 0·3 % to 0·4 % in the mutant A4-CR. Lower frequencies were observed using other mutagens. The mutants from W70 behaved similarly.

Table 2. *The composition of slime and capsule exopolysaccharides*

Strain	Type	Growth temperature	Constituents	Molar ratio (approx.)
A4	Capsule, slime	All	Glc:Gal:GlcUA	1:2:1
A4-CR	Capsule, slime	30 to 35°	Glc:Gal:GlcUA	1:2:1
A3(S1)	Slime	All	Glc:Fuc:GlcUA:acetyl	4:2:2:1
A3-CR	Slime	30 to 35°	Glc:Fuc:GlcUA:acetyl	4:2:2:1
W70	Capsule, slime	All	Gal:Mann:GlcUA:acetyl	1:1:1:1
W70-CR2	Capsule, slime	30 to 35°	Gal:Mann:GlcUA:acetyl	1:1:1:1

Exopolysaccharide synthesis

It was obvious during the initial examination of cultures that differences in exopolysaccharide synthesis occurred between parent and CR strains. It was thus possible that the mutants might synthesize polysaccharides of altered composition and structure. The repeating unit of *Klebsiella aerogenes* A3(SL) slime polysaccharide has already been identified (Sutherland, 1967; Sutherland & Wilkinson, 1968). Hydrolysates of A4 polysaccharide contained glucose, galactose and glucuronic acid, while acetyl and pyruvoyl groups were absent. The capsular polysaccharide of strain W70 contained galactose, mannose, glucuronic acid and *O*-acetyl groups. The composition and approximate molar ratios of the polysaccharides are shown in Table 2 together with the composition of the material secreted by the three corresponding CR mutants when grown at 35°. The parent and mutant strains secrete polysaccharides of the same chemotype with sugars present in the same molar ratio. Further proof of the similarity of the pairs of polysaccharides was obtained by comparison of partial acid hydrolysates by paper electrophoresis and paper chromatography. The fragments obtained were identical for each parent and mutant strain. In addition, phage-induced fucosidases active against A3(SL) polysaccharide yielded the same hydrolysis products from A3(SL) and A3-CR polysaccharides.

On nitrogen-limited defined media with glucose as carbon and energy source, the production of polysaccharide was determined after separation from cells by the anthrone technique. The slime or capsular material was removed by boiling and the bacterial deposits by centrifugation. For all the strains examined, the greatest differences between parent and mutant strains were observed at 20° at which temperature the CR mutants produced only about 30 % that excreted by the parent in liquid medium. Similar results were obtained for each set of mutants and values for A4, A4-CR and

A4-CRO are given in Table 3. The CR-O mutants produced no polysaccharide detectable by the techniques used, and so resemble O mutants.

Lipopolysaccharides and glycogen

The occurrence of autoagglutination and 'roughness' in other species of Enterobacteriaceae in usually associated with the production of incomplete lipopolysaccharides (Lüderitz, Staub & Westphal, 1966). Thus examination of the mutant lipopolysaccharides might reveal loss of part of the macromolecule. An earlier study of the A3 and A4 strains showed that they contained 17 to 23 % galactose but only 2 % glucose (Sutherland & Wilkinson, 1966). They thus differed from the types of polymer common to members of the genera *Salmonella*, *Shigella* and *Escherichia*, which all

Table 3. *Exopolysaccharide production on nitrogen-limited media*

		Incubation temperature							
		35°			30°			20°	
Strain	Medium	24 hr	48 hr	72 hr	48 hr	72 hr	96 hr	72 hr	96 hr
A4	Solid	2·8	3·5	3·0	1·6	2·0	2·8	3·7	3·7
	Liquid	2·1	2·3	2·4	2·1	1·9	2·0	2·2	2·2
A4-CR	Solid	3·3	2·1	2·6	1·4	2·0	2·5	1·0	0·9
	Liquid	2·9	2·0	2·1	1·8	2·0	2·6	1·2	1·1
A4-CRO	Solid or liquid	0·1	0·1	0·1	0·1	0·05	0·05	0·05	0·05

All values are given as mg. polysaccharide (glucose anthrone value)/mg. cell protein.

Table 4. *The yield of lipopolysaccharides by CR and CR-O strains*

			Lipopolysaccharide		
Strain	Growth temperature	Lipopoly-saccharide (% dry cells)	Glucose	Composition galactose	Glycogen (% dry cells)*
A3-CRO	20°	1·10	2·4	19·0	2·2
	35°	3·10	1·2	15·0	5·5
W70-CR2	20°	1·45	1·1	14·0	2·8
	35°	5·09	1·0	16·3	1·0
A4-CRO	20°	2·57	1·6	17·9	5·4
	35°	3·33	1·2	18·6	1·9
A4-CR	20°	1·2	2·5	22·5	6·6
	35°	2·1	3·7	20·7	6·0

* The values for glycogen were derived by multiplying the glucose content of the ultracentrifuge supernatants by the weight of dry material.

contain approximately equal quantities of glucose and galactose in their basal structures (Lüderitz *et al.* 1966). The galactan nature of the lipopolysaccharide of Klebsiella A3(SL) and another strain has been confirmed by structural studies (Koeltzow, Epley & Conrad, 1968). Strain W70 also proved to have a similar lipopolysaccharide.

Several of the CR and CR-O strains were grown in nutrient broth containing 1% (w/v) glucose in shaken culture at 20° or 35°. The bacteria were harvested by centrifugation, washed and lyophilized. The yields of lipopolysaccharide extracted by phenol/water and purified by ultracentrifugation are shown in Table 4. It can be seen that the polymer yields were generally lower following incubation at 20° than those from 35°. The composition of the macromolecules as measured by their glucose and galactose contents appeared to be constant. The yields of glycogen varied considerably and do not show any distinct pattern.

Phage sensitivity

One of the methods used to check the identity of mutants with the wild type was to test the sensitivity of both strains to one or more bacteriophages. The bacterial culture was incubated in nutrient broth then spread over the surface of plates of nutrient agar. These were allowed to dry at room temperature for 30 min. Drops (0·02 ml.) of bacteriophage suspensions containing approximately 10^6 plaque-forming units were applied and incubation continued at varying temperatures. Initial tests showed no difference in phage sensitivity after incubation at 20° or 37°. When, however, the parent cells were incubated through two subcultures in nutrient broth at 20° then used to seed the plate, there were several differences from the sensitivity pattern observed at 37°. These results are shown in Table 5. It seems probable that in

Table 5. *Phage sensitivity patterns of mutant and wild-type strains*

(+, Confluent lysis; −, no lysis; ±, very small numbers of plaques.)

	Incubation 37°, phage:								Temperature 20°, phage							
Host strain	31	33	36	33	38	40	41	42	31	33	36	37	38	40	41	42
A3(S1)	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
A3-CR	+	+	+	+	+	+	−	−	+	−	+	±	−	+	−	−
W70-CR1	−	−	−	−	−	+	+	+	−	−	−	−	−	+	−	+
W70-CR2	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
A3-CRO	−	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
W70	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
A4	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
A4-O	−	−	−	−	−	+	+	−	−	−	−	−	−	+	±	−
A4-CR	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
A4-CRO	−	−	−	−	−	+	+	−	−	−	−	−	−	+	±	−
Original Host	←		A3SL		→	A1	A4	W70								

certain of the mutants the phage receptor is no longer synthesized at the lower temperature or alternatively the mutants are unable to liberate mature phage. If the defect is one of surface properties it is presumably due to a loss of carbohydrate metabolism at a step which is also essential for exopolysaccharide synthesis. Thus strain A3-CR was no longer susceptible to phages F33 and 38, which both acted on the parent and on A3-CR at 37°. It is interesting that in some cases the mutants are susceptible to more phages than the mucoid parent. This is presumably due to occlusion of the bacterial surface by the capsule preventing phage uptake as it is not seen in the A3 mutants where the parent strain produces slime only.

DISCUSSION

Mutations involving exopolysaccharide biosynthesis in *Klebsiella aerogenes* appear to occur in two ways. The wild-type organisms producing capsules and slime may undergo a mutation causing loss of the ability to form a discrete capsule. Such a slime-forming (SL type) mutants are exemplified by *K. aerogenes* A 3 (SL) which secretes an exopolysaccharide identical in all respects tested with the capsular polysaccharide synthesized by the parent cells (Dudman & Wilkinson, 1956). Either the wild type or the SL type can undergo a mutation causing complete loss of polysaccharide-synthesizing capacity. Such non-mucoid (O) mutants show no tendency to revert. Attempts to render them mucoid by transduction were unsuccessful, although other markers were transferred (McPhee, Sutherland & Wilkinson, 1969). Such O mutants deficient in precursor-synthesizing or transferase activity are analagous to many of the mutants isolated during studies on lipopolysaccharide biosynthesis (Lüderitz *et al.* 1966). Due to the lack of suitable selection procedures no mutants synthesizing in complete exopolysaccharide have yet been identified. Indeed by virtue of their repeating unit structure it is probable that the mutation is an 'all or none' phenomenon causing complete loss of polysaccharide-synthesizing capability. Nothing analagous to the 'rough' mutants isolated in studies on lipopolysaccharide biosynthesis has yet been found in exopolysaccharide studies.

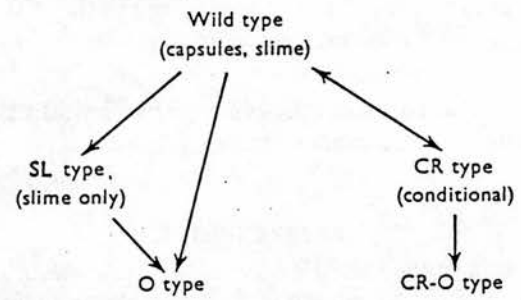

Fig. 1. Scheme for mutations involving polysaccharide synthesis in the genus *Klebsiella*.

The second and apparently unusual form of mutation is that involved in CR mutants. These apparently only arise spontaneously or under the influence of aminopurine. CR clones appear to be much less stable than O mutants. Further mutagenesis can, albeit rarely, lead to isolation of a double mutant retaining the characteristic colonial morphology of the CR mutant but having completely lost exopolysaccharide-synthesizing capacity even at 37°. The scheme involving such mutations is shown in Fig. 1. The CR mutants are obviously conditional mutants and are unusual in that the mutation appears to have two distinct effects, assuming that no double mutation has occurred. The first effect is on exopolysaccharide synthesis, which is greatly reduced by lower incubation temperature but returns to the level of the wild type on temperature up-shift. The second effect is on one of the surface components of the bacterial cells, apparently the lipopolysaccharide. This effect appears to be a quantitative one rather than an alteration to the lipopolysaccharide structure. A consequence of the lower lipopolysaccharide content is the agglutinability and 'roughness' of the cultures. These

observed results from the conditional mutation are consistent with an alteration to an enzyme involved in the formation of nucleotide precursors for the lipopolysaccharide and the exopolysaccharide or alternatively an altered regulator. The lipopolysaccharides of the Klebsiella strains used in this study contain galactose, heptose, 2-ketodeoxyoctonic acid and *N*-acetylglucosamine (Sutherland & Wilkinson, 1965). The sugars found in the slime or capsular polysaccharides are fucose, glucose, galactose glucuronic acid and mannose. Thus galactose is the only sugar common to both lipopolysaccharide and exopolysaccharide, but only in strains A4 and W70, *not* in A3(SL). We therefore conclude that the mutation occurs in the sequence of reactions.

glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose

Alteration in any of the three enzymes involved, hexokinase, phosphoglucomutase or UDP-Glc pyrophosphorylase would affect either polymer if UDP-Glc is the glucosyl donor involved in exopolysaccharide biosynthesis. The results for glycogen yields were not consistent enough to determine whether a significant reduction had occurred at lower temperatures. The donor for glycogen synthesis being ADP-glucose (Gahan & Conrad, 1968), a reduction in glycogen would favour the first two enyzmes in the sequence as the possible sites of mutation. One similar conditional alteration in surface property involving what is also probably a polysaccharide, has been reported (Knolle & Ørskov, 1967). The f+ antigen of *Escherichia coli* is lost at lowered incubation temperature but is again formed on return of the cells to 37°.

Further experiments to identify the enzyme involved in the 'CR' conditional mutation of *Klebsiella aerogenes* strains are proceeding.

The authors are grateful to Miss I. C. Miller for technical assistance and to the Science Research Council for financial support.

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Printed in Great Britain

The Synthesis of Exopolysaccharide by *Klebsiella aerogenes* Membrane Preparations and the Involvement of Lipid Intermediates

By I. W. SUTHERLAND and MARY NORVAL*
Department of General Microbiology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JG, U.K.

(*Received* 20 *July* 1970)

1. Membrane preparations from *Klebsiella aerogenes* type 8 were shown to transfer glucose and galactose from their uridine diphosphate derivatives to a lipid and to polymer. The ratio of glucose to galactose transfer in both cases was 1:2. This is the same ratio in which these sugars occur in native polysaccharide. Galactose transfer was dependent on prior glucosylation of the lipid. Mutants were obtained lacking (*a*) glucosyltransferase and (*b*) galactosyltransferase. The transferase activities in a number of non-mucoid mutants was examined. 2. Glucose transfer was partially inhibited by uridine monophosphate, and incorporation of either glucose or galactose into lipid was decreased in the presence of uridine diphosphate. The sugars are thought to be linked to a lipid through a pyrophosphate bond, and treatment of the lipid intermediates with phenol yielded water-soluble compounds. These could be dephosphorylated with alkaline phosphatase. Transfer of glucuronic acid to lipid or polymer from uridine diphosphate glucuronic acid was much lower than that of the other two sugars. 3. The fate of sugars incorporated into polymer was also followed. Some conversion of glucose into galactose and glucuronic acid occurred. Mutants unable to transfer glucose or galactose to lipid were unable to form polymer. Other mutants capable of lipid glycosylation were in some cases unable to form polymer. A model for capsular polysaccharide synthesis is proposed and its similarity to the formation of other polymers outside the cell membrane is discussed.

Two polysaccharides common to most Gram-negative bacteria, mucopeptide and lipopolysaccharide, both contain repeating units in their structure. The synthesis of each of these compounds, reviewed by Ghuysen, Tipper & Strominger (1969) and by Nikaido (1968) respectively, occurs by membrane-bound enzymes. Sugars are added sequentially from sugar nucleotide precursors to a lipid carrier. The lipid involved is apparently very similar in each system and has been identified as a phosphorylated C_{55}-isoprenoid alcohol of the general structure: $-[CH_2-C(CH_3)=CH-CH_2]-$ (Wright, Dankert, Fennessey & Robbins, 1967; Higashi, Strominger & Sweeley, 1967). The Gram-positive species *Micrococcus lysodeikticus* similarly uses a lipid carrier during synthesis of an extracellular mannan (Scher, Lennarz & Sweeley, 1968).

A third type of extracellular polysaccharide synthesized by Gram-negative bacteria, and found also in some Gram-positive species, is capsule or slime material. The synthesis of this has received much less attention than that of other polymers. Studies with *Diplococcus pneumoniae* showed the requirement for particulate enzyme preparations and the incorporation of radioactively labelled sugars from appropriate sugar nucleotides into polymer (Smith, Mills & Bernheimer, 1961). More recent work on the structure of a number of capsular or slime polysaccharides from Gram-negative bacteria showed that they are generally composed of fairly simple repeating units composed of three to six sugar residues (e.g. Hungerer, Jann, Jann, Ørskov & Ørskov, 1967; Conrad, Bamburg, Epley & Kindt, 1966; Sutherland, 1969). There is thus some similarity between the O-antigen component of lipopolysaccharides and the exopolysaccharides synthesized by such species as *Escherichia coli* or *Klebsiella aerogenes*. Both are extracellular polymers and both contain sugar repeating units of grossly similar type. The only indication of similarities in the biosynthesis of these two polysaccharides was the report of involvement of a lipid carrier stage in the synthesis of capsular polysaccharide by a *K. aerogenes* strain (Troy & Heath, 1968). The component sugars of the polymer were galactose, mannose and glucuronic acid, but no details of the structure were given.

The strain A4 (serotype 8) of *K. aerogenes* has a simple tetrasaccharide repeating unit (Fig. 1) (Sutherland, 1970). It does not contain acetyl or

* Present address: Department of Pharmacology, University of Wisconsin, Madison, Wis., U.S.A.

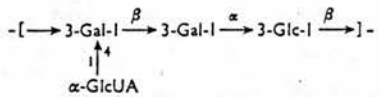


Fig. 1. Tetrasaccharide repeating unit of *K. aerogenes* strain A4 capsular polysaccharide.

pyruvyl groups and was chosen for its simple structure and the ready availability of the presumed glycosyl donors: UDP-glucose, UDP-galactose and UDP-glucuronic acid. An examination of several of the enzymes thought to be involved in the formation of the sugar nucleotide glycosyl donors required for exopolysaccharide synthesis has already been made (M. Norval & I. W. Sutherland, unpublished results) with the wild-type strain and a number of mutants. The present study reports the use of membrane preparations in a cell-free system for the transfer of sugars from sugar nucleotides to lipid-soluble material and to polysaccharide-resembling capsular material.

MATERIALS AND METHODS

Cultures. The wild-type strain of *K. aerogenes* strain A4 (serotype 8) was studied by Dudman & Wilkinson (1956) and the structure of its exopolysaccharide capsule has been determined (Sutherland, 1970). The isolation of mutants from it and the contents of several of the enzymes involved in sugar nucleotide synthesis have been examined (M. Norval & I. W. Sutherland, unpublished work). Bacteria were normally grown in nutrient broth or in trypticase soy broth (Baltimore Biologicals Laboratory, Baltimore, Md., U.S.A.) supplemented by 1% (w/v) glucose. A number of mutants were also grown in nutrient broth containing 1% (w/v) galactose. Cultures were grown in 1 litre quantities in 2-litre Erlenmeyer flasks shaken at 35°C for 15h at 200rev./min.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Glucose, galactose and glucuronic acid were determined as described by Sutherland (1970).

Chemicals. Unlabelled sugar nucleotides were purchased from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.) and from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). ^{14}C-labelled sugar nucleotides (randomly labelled in the sugar moieties) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Other chemicals and biochemicals were of the highest grade commercially available.

Chromatography and electrophoresis. Descending paper chromatography was performed at room temperature in the following solvent systems with Whatman no. 1 paper: solvent A, butan-1-ol–pyridine–water (6:4:3, by vol.) (Whistler & Conrad, 1954); solvent B, ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (Feather & Whistler, 1962); solvent C, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) (Fischer & Doerfel, 1955); solvent D, ethanol–1.0M-ammonium acetate (pH7.5) (7:3, v/v) (Paladini & Leloir, 1952). T.l.c. on silica gel G plates was carried out in solvent E [chloroform–methanol–water (12:6:1, by vol.)]. Paper electrophoresis on Whatman no. 1 paper was performed in pyridine–acetic acid buffer, pH5.3. Locarte paper-electrophoresis equipment was used with a current of 90mA applied for 3h at 40V/cm. Development of chromatograms or electrophoresis strips was carried out with alkaline $AgNO_3$ reagent or with the phosphate reagent of Hanes & Isherwood (1949).

Measurement of radioactivity. Radioactivity on paper strips was located with a Tracerlab 4 pi Scanner. Because of the low efficiency of counting, results were usually checked by cutting out 1cm segments and placing them in vials with 10ml of toluene containing 0.5% of 2,5-diphenyloxazole for scintillation counting in a Beckmann ambient-temperature scintillation spectrometer (Lennarz & Talamo, 1966). Lipid extracts were evaporated to dryness at 60°C and their radioactivities counted in 10ml of Triton scintillation fluid (Lennarz & Talamo, 1966). Radioactivities of material from t.l.c. plates were also counted in this fluid. For aqueous samples a dioxan-based scintillation fluid was used [Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.]. The efficiency of counting for ^{14}C was approx. 50% for liquid samples and 30% for paper samples determined by scintillation counting.

Preparation of membrane material. Cells from 2 litres of culture were harvested by centrifugation at 5000*g* and washed twice in 0.9% NaCl buffered to pH8.0 with 10mM-tris. They were then resuspended in 50ml of 10mM-tris–HCl buffer, pH8.0, and 10ml portions were treated for 2min in MSE ultrasonic equipment with 100W output. The container was cooled with an ethanol–ice mixture and the unbroken cells were removed by centrifugation at 5000*g* for 20min. The supernatant fluid from this preparation was then centrifuged at 20000*g* for 60min and washed by resuspension in tris buffer. The reddish gel of membrane material was carefully removed and resuspended in 5ml of water at 0°C.

Assay for lipid material. Samples (50μl) from incubation mixtures were added to 4ml of chloroform–methanol (2:1, v/v) and homogenized in a vortex mixer. The tubes were then placed in a water bath at 60°C for 5min and again mixed. Then 1ml of 0.9% NaCl was added and the mixture was again homogenized. Phase separation was achieved by centrifugation for 15min in a bench centrifuge. The aqueous layer and interface were carefully removed by using a long Pasteur pipette and gentle suction. The chloroform layer was carefully transferred to scintillation vials with fresh Pasteur pipettes. Zero-time controls consistently showed radioactivities of 20–25 c.p.m. This was also true of samples of the different labelled sugar nucleotides included to check that there was no breakdown or carry-over of sugars with this technique.

Assay for polymer material. Samples (25 or 50μl) from incubation mixtures were applied to Whatman no. 1 paper as 2cm strips. The sheets of paper were irrigated for 20h in solvent D to remove low-molecular-weight material. The areas at the origin forming strips 2cm×1cm were cut out and their radioactivities determined.

RESULTS

Transfer of sugars to lipid material. The ability of osmotically shocked cells of *K. aerogenes* to

transfer sugars from sugar nucleotides to lipid material was observed by Troy & Heath (1968). There are, however, considerable disadvantages in using whole cells. In an attempt to overcome these, membrane material was prepared as described in the Materials and Methods section. This was then used as a potential source of enzymes for exopolysaccharide synthesis. Incubation mixtures were prepared as follows: 0.25M-tris–HCl buffer, pH 8.0, 100 μl; 50mM-magnesium chloride, 50 μl; membrane suspension, 200 μl; water, 100 μl. Sugar nucleotides were added in the same proportions as the sugars were found in the polysaccharide, namely 400nmol of UDP-glucose or UDP-glucuronic acid and 800nmol of UDP-galactose. Labelled nucleotides were added as follows: UDP-glucose, 3.7×10^5 c.p.m.; UDP-galactose, 1.1×10^5 c.p.m.; UDP-glucuronic acid, 5.5×10^5 c.p.m. The total volume was adjusted to 550 μl with water. The mixtures were allowed to equilibrate to room temperature (20°C) before addition of the sugar nucleotides and finally the membrane.

When single nucleotides were added, with membrane from wild-type cells, there was rapid transfer of glucose or galactose from their respective sugar nucleotides to lipid material but no transfer of glucuronic acid. The rate of incorporation of either sugar was maximal over the first 10min but continued at a decreased rate for 30–40min. Typical results are shown in Fig. 2. After 30min the ratio of glucose incorporation to galactose incorporation was approx. 3.5:1, and no glucuronic acid was transferred.

On the addition of all three sugar nucleotides, each in turn being labelled, a different pattern of incorporation was observed. Typical results (Fig. 3) show that the amount of galactose incorporated into lipid is approximately twice that of glucose. Much less glucuronic acid than glucose or galactose was transferred. As the cells contain an active UDP-galactose 4-epimerase in the membrane fraction (M. Norval & I. W. Sutherland, unpublished work) it was to be expected that some interconversion of the sugars would occur. Such interconversion would only be absent from epimerase-less mutants and attempts to obtain these were unsuccessful. The presence of a particulate UDP-glucose dehydrogenase would also permit some incorporation of glucose into UDP-glucuronic acid and hence into lipid, but the very small extent of transfer of labelled glucuronic acid from UDP-glucuronic acid to lipid was unexpected. The absence of such transfer activity might be due to: (i) large quantities of membrane-bound UDP-glucuronic acid; (ii) the addition of glucuronic acid from a precursor other than UDP-glucuronic acid; (iii) a soluble glucuronyltransferase; or (iv) the addition of glucuronic acid at a relatively late stage in biosynthesis to a neutral sugar 'core'. The first three of these possibilities can be excluded. Extraction of membrane preparations with ethanol yielded only trace amounts of UDP-glucuronic acid, identified and determined as described by Grant, Sutherland & Wilkinson (1970). Nor was any other glucuronic acid-containing nucleotide detected. The addition to the assay mixture of the 20000*g* supernatant obtained during membrane preparation caused no increase in glucuronic acid transfer to lipid.

Transfer activity. Attempts to obtain transfer of the neutral sugars to lipid material were made with

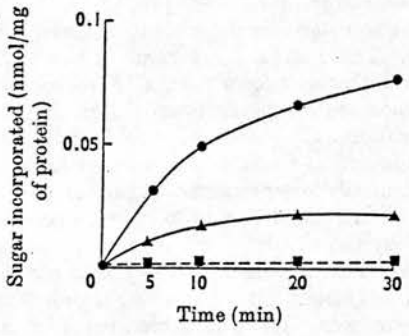


Fig. 2. Transfer of sugar from sugar nucleotides to lipid. The standard incubation mixture was used containing a single sugar nucleotide together with ^{14}C-labelled material. Samples (50 μl) were withdrawn at intervals and extracted (400nmol of UDP-glucose or UDP-glucuronic acid or 800nmol of UDP-galactose was used) with chloroform–methanol (2:1, v/v). ●, UDP-glucose; ▲, UDP-galactose; ■, UDP-glucuronic acid.

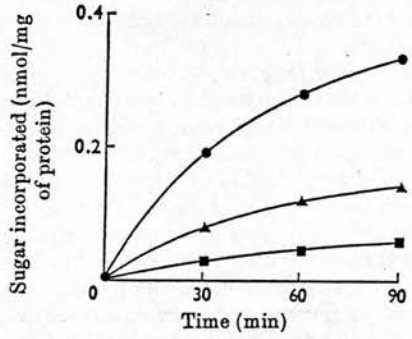


Fig. 3. Transfer of sugars from mixtures of sugar nucleotides to lipid. The standard incubation mixture contained, in each case, all three sugar nucleotides, one of which was labelled with ^{14}C in the sugar moiety. Samples were taken periodically for chloroform–methanol extraction. ●, UDP-galactose; ▲, UDP-glucose; ■, UDP-glucuronic acid.

a number of cell fractions. Only a complete ultrasonic lysate and the material deposited by centrifugation at 20000*g* were active in the transfer of glucose or galactose. On the basis of protein content the membrane preparations were two to three times as effective as an equivalent amount of whole-cell lysate in the transfer of either sugar from the corresponding sugar nucleotide to lipid material. The supernatant fluid from the 20000*g* centrifugation and material deposited from it by ultracentrifugation at 100000*g* were completely inactive.

Normally membrane preparations were used immediately after preparation, all stages during the process being performed in the cold. However, preparations held in vials completely surrounded by ice at 0°C for up to 4 days retained 90% of their activity. Preparations that were frozen and then rapidly thawed at room temperature lost all transferase activity. When thawing was carried out slowly over a period of 4–5h frozen samples retained about 80% of their activity with respect to glucose or galactose transfer. This was true of material kept for up to 28 days at −20°C.

A requirement for Mg^{2+} has been shown for glycosyltransferase activity in other polysaccharide-synthesizing systems, and magnesium chloride was accordingly included in the present assay system. Omission of Mg^{2+} resulted in decrease of glucose transfer by about 25%. It is, however, difficult to wash membrane preparations thoroughly with water, as the presence of mucoid material from wild-type cells leads to a loosely packed sediment that probably carries over ions from the original culture. Consequently the validity of this result is difficult to assess. Replacement of Mg^{2+} with other bivalent cations was also tested. The presence of Co^{2+} or Ca^{2+} ions resulted in the same extent of glucose transfer but Mn^{2+} caused a 30% decrease. This could be due to competition for sites normally using Mg^{2+}.

Transferase activity in mutants. A number of mutants unable to synthesize capsular polysaccharide were isolated. Examination of 23 of these non-mucoid mutants showed that when membrane preparations were incubated with the three sugar nucleotides transfer of glucose or galactose to lipid was lower than in wild-type preparations. Results are exemplified by strains 030, 032, 034 and 036 shown in Table 1. In all of these mutants tested, the ratio of glucose to galactose incorporation into lipid resembled that of wild-type material in having a value of almost 1:2. In only two mutants (040 and 042) of this group were deletions known to occur before the sugar nucleotide stage. These two mutants were defective in phosphoglucomutase and UDP-glucose pyrophosphorylase respectively (M. Norval & I. W. Sutherland, unpublished work). The other 21 strains had normal profiles for the enzymes leading to sugar nucleotide formation and modification. Four further mutants (K119, K121, 029 and 038) incorporated glucose but not galactose. Repeated experiments confirmed this result. Two mutants (K138 and 037) showed no incorporation of either glucose or galactose into lipid. When equal quantities of membrane preparations from strain 037 and either strain 029 or strain 038 were combined, incorporation of glucose was detected together with some incorporation of galactose. This indicated that some galactose transferase activity was present in the strain-037 preparation and that galactose transfer was dependent on prior glucose transfer. The results for several of these strains together with those for a normal *K. aerogenes* A4 preparation for comparison are shown in Table 1. Incorporation of glucuronic acid from UDP-glucuronic acid was too low to permit accurate assay.

Table 1. *Comparison of sugar incorporation into lipid material by wild-type and mutant strains*

The standard incubation mixture containing UDP-glucose, UDP-galactose and UDP-glucuronic acid was used with either UDP-[^{14}C]glucose or UDP-[^{14}C]galactose. Samples were withdrawn at 30 min intervals and extracted with chloroform–methanol (2:1, v/v). The resultant incorporation is based on the values obtained at 60 min after the start of incubation.

	Sugar incorporation (nmol/h per mg of membrane protein)	
Strain	Glucose	Galactose
Wild-type	0.124	0.284
030	0.010	0.019
032	0.046	0.068
034	0.053	0.090
036	0.053	0.124
037	0	0
029	0.013	0
038	0.020	0
037 + 038	0.009*	0.004

* Results expressed relative to total protein.

The generally lower extent of sugar transfer in the mutant strains could be due to lowered production of the enzymes involved, perhaps through specialized control mechanisms. To test this membrane preparations from the phosphoglucomutase-less strain were used. The cells were grown (*a*) in the trypticase soy medium supplemented with glucose and (*b*) in the same medium supplemented with galactose. The results for sugar transfer obtained with the two preparations are shown in Table 2. In this strain at least there is much lower transferase activity with membranes prepared from cells in the glucose medium, i.e. conditions under which no exopolysaccharide is synthesized.

Effects of potential inhibitors. In other systems in which transfer of sugars to lipid occurs a number of inhibitors are known. Thus the detergent Triton X-100 selectively prevents mannan synthesis from mannolipid in *M. lysodeikticus* (Scher & Lennarz, 1969) and bacitracin stops phosphate release in the final stage of mucopeptide synthesis (Siewert & Strominger, 1967). If the process of exopolysaccharide synthesis is similar to these other systems such inhibitors might also be effective. A membrane preparation from wild-type cells was used and the results of Triton and bacitracin on sugar transfer to lipid are shown in Table 3. Unlike the effect on the mannan system, Triton inhibits transfer of sugars to lipid. Bacitracin also affects sugar transfer to some extent, thus resembling the effect on mucopeptide system but differing from that on lipopolysaccharide biosynthesis.

The mechanism of transfer of the initial sugar in O-antigen synthesis involves transfer of the sugar 1-phosphate together with release of the nucleotide phosphate. Thus, if the first step in capsule polysaccharide formation is the transfer of glucose 1-phosphate from UDP-glucose, UMP should be released and it should also inhibit the reaction. Similarly subsequent sugar transfer might be inhibited by UDP. The standard assay system for glucose incorporation was first used with UDP-glucose as the only sugar nucleotide present. Similarly the effect on galactose transfer from a mixture of UDP-glucose and UDP-galactose was also tested. The results for these mixtures are shown in Figs. 4(*a*) and 4(*b*) respectively. Clearly the transfer of glucose to lipid is strongly inhibited by the presence of UMP. The effect of UDP is probably in stopping transfer of galactose from UDP-galactose, formed by epimerization of the UDP-glucose. The almost complete inhibition of galactosyl transfer by UDP

Table 2. *Activity of membrane preparations from a phosphoglucomutase-less mutant (strain 040) grown in media supplemented with glucose or galactose*

The standard incubation mixture with all sugar nucleotides was used. Samples were withdrawn at 30min intervals and extracted with chloroform–methanol (2:1, v/v).

	Sugar incorporation (nmol/h per mg of protein)	
Medium containing	Glucose	Galactose
Glucose	0.006	0.006
Galactose	0.031	0.090

Table 3. *Effect of bacitracin or Triton X-100 on sugar transfer to lipid*

The incubation mixture in all experiments contained all three sugar nucleotides together with either UDP-[^{14}C]-glucose or UDP-[^{14}C]galactose. Samples were extracted with chloroform–methanol (2:1, v/v) at 30min intervals and the values for sugar incorporation are based on the 60min value.

	Sugar incorporation (nmol/h per mg of protein)	
Incubation mixture	Glucose	Galactose
Complete	0.246	0.405
Complete+250μg of bacitracin	0.170	0.311
Complete+0.25% Triton X-100 (final concn.)	0.150	0.141

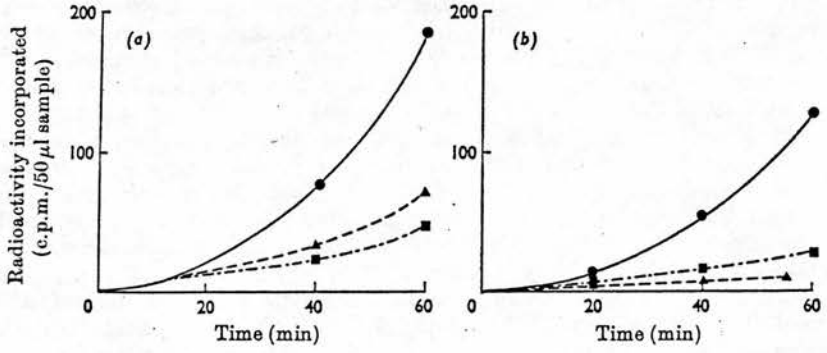


Fig. 4. Effect of UDP and UMP on sugar transfer to lipid. The incubation mixture with UDP-glucose only was used in (*a*) to show the effect on glucose transfer of the addition of UDP (200nmol) or UMP (200nmol). Samples were taken at intervals and extracted with chloroform–methanol. In (*b*) the effect of the same concentrations of UDP or UMP on galactose transfer from a mixture of UDP-glucose and UDP-galactose is shown. ●, Complete system; ▲, +UDP; ■, +UMP.

is shown in Fig. 4(*b*), whereas the extent of inhibition by UMP corresponds to that expected from the results for UDP-glucose. As a further check on the initial reaction, involving glucose transfer, a series of ten standard incubation mixtures containing UDP-glucose only was prepared. After 30 min of incubation at 20°C with *K. aerogenes* A4 membrane the reactions were terminated by pouring the suspensions into boiling ethanol. Extraction of nucleotides was performed as described by Grant *et al.* (1970). The water-soluble nucleotides were separated by paper electrophoresis for 4h and identified by staining with molybdate reagent (Hanes & Isherwood, 1949). Only two major spots were detected, corresponding in their electrophoretic mobilities to UDP-glucose and UMP respectively. A very small amount of material moving ahead of UDP-glucose was possibly UDP. A control experiment in which no membrane was added to the incubation mixture showed UDP-glucose only.

Characterization of lipid-soluble material. Attempts to scale up the incubation mixture to obtain sufficient glycosyl-lipid were not satisfactory. Better results were obtained by pooling material from a number of experiments and using the standard incubation mixture. The chloroform–methanol-soluble material was obtained in the normal manner except that extraction was at room temperature for 15 min. The chloroform layers were then pooled, back-washed with water and evaporated under reduced pressure. The residual material was extracted into a small volume of methanol. The recovery of radioactive material was approx. 90% of that originally present in the chloroform layers. Such material was prepared from incubation mixtures with either UDP-glucose as the sole sugar nucleotide or complete mixtures with each sugar in turn labelled. On electrophoresis in pyridine–acetic acid buffer, pH 5.3, all radioactivity remained at the origin. Chromatography in solvent D or t.l.c. on silica gel yielded some separation. In solvent D chromatograms of lipid labelled from either UDP-glucose or UDP-galactose showed two main areas of radioactivity. A broad band with R_{Gal} 1.26–1.34 contained 70% of the radioactivity detected. A further 15% moved with R_{Gal} 1.09, and three other peaks at R_{Gal} 0.65, 0.48 and 0.22 accounted for the remaining radioactivity. On the basis of comparison with UDP-glucose or UDP-galactose run under the same conditions, the peaks with R_{Gal} 0.65 and 0.48 are probably cyclic phosphates, which occur as artifacts under the chromatographic conditions used (Tovey & Roberts, 1970). When UDP-[^{14}C]glucuronic acid was used two areas of radioactivity with R_{Gal} 1.26 and 0.13 were obtained. These possibly correspond to lipid-bound tetrasaccharide and tetrasaccharide phosphate respectively. On t.l.c. in solvent E material labelled with glucose or galactose showed two peaks with R_F 0.63 and 0.25 respectively, about 80% of the label being in the slower-moving peak. This is thus similar in its mobility to the mannolipid described by Caccam, Jackson & Eylar (1969). Insufficient glucuronic acid-labelled material was available to allow accurate identification of peaks.

Phenol treatment. The pyrophosphate bridge of sugar diphosphate–lipid complexes can be broken by treatment with 45% (v/v) phenol at 60°C for 5 min (Kent & Osborn, 1968). This method was used to examine the sugar diphosphate–lipid complexes formed by incubation of the sugar nucleotides with *K. aerogenes* membrane preparations. The lipid material was dissolved in 50 μl of methanol and added to 450 μl of water, and 500 μl of aq. 90% (v/v) phenol was added. After 5 min at 60°C the mixture was cooled and extracted twice with 2 ml portions of ether. Aqueous layers were freeze-dried. Samples of both organic and aqueous layers were checked for radioactivity, which was all found in the aqueous layers. Samples from the aqueous layers were submitted to paper electrophoresis. Little difference was detected in the products obtained with UDP-[^{14}C]glucose or UDP-[^{14}C]galactose in a complete mixture of nucleotides. In each case the only radioactive material detected moved in a broad band with M_{GlcUA} 0.93–0.95 (cf. glucose 6-phosphate, M_{GlcUA} 0.65). After treatment with alkaline phosphate for 1h at 37°C under conditions where 1 mmol of glucose 1-phosphate was completely dephosphorylated, the preparations were again subjected to paper electrophoresis. All radioactivity was now detected in three areas, M_{GlcUA} 0.60 and 0.45 and at the origin. The electrophoretic mobilities correspond to those obtained for trisaccharides and a tetrasaccharide obtained by partial acid hydrolysis of *K. aerogenes* A4 polysaccharide and included on the paper for comparison (Sutherland, 1970). When UDP-glucose was the sole sugar nucleotide present and the reaction was stopped after 30 min at room temperature, the product of phenol treatment was slightly different. Two areas of radioactivity were detected after electrophoresis (M_{GlcUA} 0.65 and 0.60). After phosphatase treatment all radioactivity remained at the origin.

When the products of phenol treatment were incubated with alkaline phosphatase and applied to chromatograms in solvent B, the results from mixtures containing all nucleotides and labelled with UDP-[^{14}C]glucose or UDP-[^{14}C]galactose were similar. Each showed large amounts of radioactivity with R_{Glc} 0.05 and 0.10, equidistant with a trisaccharide and a tetrasaccharide isolated previously from *K. aerogenes* A4 polysaccharide by partial acid hydrolysis (Sutherland, 1970). A small amount of material equidistant with glucose was observed. Another major peak of radioactivity had R_{Glc} 0.35.

This is probably the disaccharide *O*-α-D-galactosyl-(1→3)-D-glucose, as lactose and melibiose, the only galactosides available for comparison, had R_{Glc} values 0.32 and 0.30 respectively in this solvent. The product obtained when UDP-glucose was the sole sugar nucleotide present in the reaction mixture revealed only radioactivity equidistant with glucose and with the presumed disaccharide. These results strongly suggested that the sugars were being incorporated into the lipid in the same configuration in which they are present in the polysaccharide. The products from the UDP-glucose incorporation would thus be glucose 1-phosphate and galactosylglucose 1-phosphate. Insufficient glucuronyl-labelled material was available to permit comparison with the other products.

Polymeric material. No specific enzymes hydrolysing *K. aerogenes* serotype 8 exopolysaccharide are known. Consequently assay methods for the polymer are very limited. That described in the Materials and Methods section was used as a routine for convenience, but it is non-specific. A second, specific, method was used to confirm these results. This utilized the observation (Sutherland, 1970) that mild acid hydrolysis (0.25M-sulphuric acid at 100°C for 30min) of the serotype 8 polysaccharide yielded a number of oligosaccharides that could be separated by paper electrophoresis or by chromatography in solvent B. In all cases where polymer synthesis was shown by the first method it was confirmed by scanning after paper electrophoresis of partial acid hydrolysates. All radioactivity was found to coincide with the oligosaccharides or with neutral material.

When wild-type membrane preparations were incubated with UDP-glucose a small amount of sugar was incorporated into polymer. This was to be expected as the UDP-glucose could be converted into UDP-galactose and UDP-glucuronic acid. However, incubation with UDP-galactose or UDP-glucuronic acid failed to yield polymer. When a complete mixture of nucleotides was incubated with the membrane, polymer, as determined by both assay methods, was formed. Typical results for polymer formation, as determined by the non-specific assay with each sugar nucleotide in turn in the mixture labelled, are shown in Fig. 5. It is clear that both glucose and galactose are incorporated to a considerable extent and that the ratio of glucose to galactose incorporation is approx. 1:2. Much less glucuronic acid was incorporated. The pattern is thus very similar to that obtained for transfer of sugar to lipid.

Examination of the various mutants showed that in most cases little if any polymer was formed. This was to be expected in those mutants from which the enzymes responsible for lipid intermediate formation were known to be absent or in those which were not known to have defects in nucleotide synthesis or in lipid transferases. In these it was inferred that failure to synthesize capsule must be due either to lack of glucuronic acid transfer or to some later stage in synthesis. However, when the mutants lacking UDP-glucose pyrophosphorylase and phosphoglucomutase were examined a rather surprising result was obtained. The pyrophosphorylase-less mutant grown either on glucose or galactose media formed polymer to much the same extent as the wild-type strain. This was also true for the phosphoglucomutase-less strain when grown on media containing galactose. After growth on glucose sugar transfer to lipid occurred but no polymer was formed. The reason for this remains unclear. It is possible that under these conditions some acceptor required for polymer formation is absent and synthesis thus stops at the lipid intermediate stage. The mutant was obtained by using ethyl methanesulphonate as mutagen and as far as can be determined it is not a double mutant.

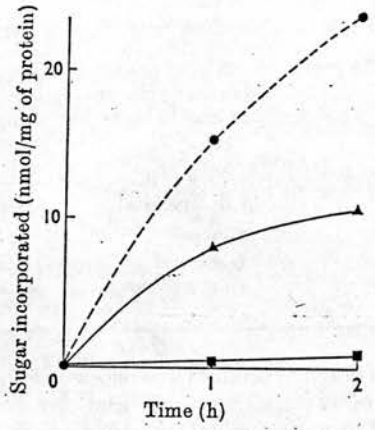


Fig. 5. Transfer of sugars from sugar nucleotides to polymer. The complete incubation mixture containing all three sugar nucleotides was used with one of them labelled in the sugar moiety in each case. Samples (25μl) were withdrawn at intervals and applied to a 2cm strip on a sheet of Whatman no. 1 paper. This was irrigated overnight with solvent D. The area of the origin was cut out, placed in scintillation fluid in a counting vial and residual radioactivity was determined. ●, UDP-galactose; ▲, UDP-glucose; ■, UDP-glucuronic acid.

Fate of label transferred to polymer. Polymer was prepared from pooled incorporation experiments in which each sugar nucleotide in turn was labelled. The starting material was the aqueous layer after chloroform–methanol extraction. Exopolysaccharide was isolated either by phenol extraction followed by dialysis and ultracentrifugation to remove sugar phosphates and lipopolysaccharide respectively, or by precipitation with 5 vol. of cold acetone.

Table 4. *Distribution of* ^{14}C *in polymer labelled with* ^{14}C*-labelled sugars from sugar nucleotides*

The polymeric material prepared by incubation of membrane with complex mixtures of sugar nucleotides, one being labelled in each case, was prepared as described in the text. Hydrolysis and chromatographic separation were then performed also as described in the text.

	Distribution of radioactivity (c.p.m.)		
Initial label	Glucose	Galactose	Glucuronic acid+lactone
Glucose	2221	193	79
Galactose	269	1239	45
Glucuronic acid	247	163	2127

In either case the product was redissolved in a small volume of water, exhaustively dialysed and freeze-dried. The crude products in each preparation were dissolved in water to give approx. 5000 c.p.m. in 200 μl. An equal volume of M-sulphuric acid was added and the samples were hydrolysed in sealed tubes for 16 h at 100°C. After neutralization with saturated barium hydroxide solution, salts and charged material were separated by paper electrophoresis, each preparation being applied to a 6 cm strip on the paper. From each paper 2 cm guide strips were cut out and checked for radioactivity. All radioactive material was located in each preparation equidistant with glucuronic acid and with the neutral material at the origin. These areas on the remaining 4 cm strips were eluted, pooled and re-run on paper chromatograms in solvent A. The areas corresponding to glucuronic acid and lactone, glucose and galactose were cut out and their radioactivities counted. The distribution of radioactivity is shown in Table 4.

If the polymer is identical with serotype 8 polysaccharide, the same products of partial acid hydrolysis should be obtained. This was performed as for the specific assay described above. After neutralization the samples were applied to Whatman no. 1 paper and subjected to paper electrophoresis. The material between glucuronic acid and the neutral material was eluted as one fraction and the neutral material as a second. They were applied to separate paper chromatograms run in solvents B and A respectively. In solvent B all material ran equidistant with the oligosaccharides isolated earlier from serotype 8 polysaccharide (Sutherland, 1970) and used as standards. No radioactivity equidistant with glucuronic acid was detected. This confirms that the conditions of hydrolysis were insufficient to hydrolyse the aldobiouronic acid *O*-α-D-glucuronosyl-D-galactose. The chromatogram of the neutral material showed that all the radioactivity ran equidistant with glucose and galactose. This is also similar to the pattern with authentic serotype 8 polysaccharide, which fails to yield any neutral oligosaccharides on partial acid hydrolysis.

DISCUSSION

The gross similarity in the structure of the repeating units of exopolysaccharide capsules of *K. aerogenes* and the O-antigenic components of lipopolysaccharides indicated a possible similarity in their synthetic processes. The fact that both polymers are located outside the cell membrane also points to possible similarities in precursor synthesis, organization, control and polymerization. The O-antigens are synthesized by particulate enzymes contained in the cell membrane. Sequential transfer of sugars from sugar nucleotide precursors to an isoprenoid alcohol phosphate acceptor occurs (Nikaido, 1968).

The results obtained in the present study showed that transfer of glucose from UDP-glucose to a lipid acceptor occurs in membrane preparations of *K. aerogenes* serotype 8. The transfer of galactose occurs similarly. The sequence is most probably glucosyl transfer followed by galactosyl transfer. This is indicated on the basis of two observations: (i) although some transfer of galactose occurred in the absence of sugar nucleotides other than UDP-galactose, the highest yields of galactose-labelled lipid were obtained when UDP-glucose was also present; (ii) mutants were obtained that transferred (*a*) neither glucose nor galactose and (*b*) glucose only; no mutants transferring galactose only were obtained. In the type of mutant unable to transfer glucose galactosyltransferase activity was present. Galactosyltransferase thus requires glucosyl transfer as a prerequisite.

The first stage in glycosylation of the lipid is probably similar to the first stage occurring in the synthesis of other extracellular polymers. In this reaction glucose 1-phosphate is transferred from UDP-glucose to the lipid carrier together with release of UMP. This is based on the inhibition of glucose transfer by UMP and identification of UMP as the major nucleotide product of interaction between UDP-glucose and the membrane-bound enzyme system. The ratio of glucose to galactose transfer in both lipid and polymer was 1:2, the same ratio in which these

sugars occur in the native exopolysaccharide. Although not conclusive, this suggests that the system is in fact one concerned with exopolysaccharide synthesis and not with some other polymer such as lipopolysaccharide from which glucose is essentially absent (Sutherland & Wilkinson, 1966). Those mutants unable to transfer glucose or galactose to lipid did not form polysaccharide, indicating that the glycosylated lipids are intermediates in exopolysaccharide formation. Further, in following the fate of the labelled sugars, radioactive material corresponding in electrophoretic and chromatographic properties to characteristic oligosaccharide components of the exopolysaccharide (Sutherland, 1970) was shown.

The lipid acceptor has not yet been characterized. Initial experiments indicated that glycosyl-labelled lipid had several properties common to the isoprenoid intermediates known to occur in the synthesis of three polysaccharides discovered outwith the bacterial cell: lipopolysaccharide (Wright *et al.* 1967), mucopeptide (Higashi *et al.* 1967) and mannan (Scher *et al.* 1968). Chromatographic results also compared well with those for a similar exopolysaccharide system studied by Troy & Heath (1968; F. C. Troy, personal communication). If the lipid is indeed an isoprenoid alcohol such compounds appear to be involved generally in the synthesis of polysaccharides outside cell membranes. This activity has also been noted in kidney cells synthesizing mannose-containing glycoproteins (Caccam *et al.* 1969). They are thus not confined to procaryotic cells in this function.

Interchangeability of lipid acceptors between mannan and exopolysaccharide systems (F. Frerman, E. C. Heath, M. Lahav, T. C. Chiu & W. J. Lennarz, unpublished work, cited by Lahav, Chiu & Lennarz, 1969) has been reported. It is not clear whether the lipids are identical in these and similar systems or merely show sufficient similarity to function in either system. If the former, the cell must possess suitable control mechanisms whereby lipid is allocated to the biosynthetic processes requiring it. It is perhaps significant that in *K. aerogenes* both UDP-galactose 4-epimerase and UDP-glucose dehydrogenase are membrane-bound (M. Norval & I. W. Sutherland, unpublished work). Thus for synthesis of polysaccharides outside the cell membrane UDP-glucose formed intracellularly would be passed to the modifying enzymes within the membrane. From these it could be spatially passed to the particle-bound enzymes and lipid carriers for the systems requiring them: exopolysaccharide with UDP-glucuronic acid and both exopolysaccharide and lipopolysaccharide with UDP-galactose. This is shown diagrammatically in Scheme 1. This could explain the failure to incorporate significant amounts of glucuronic acid from UDP-glucuronic acid. The spatial configuration of the enzymes may only accept UDP-glucose, modify it and in this way lead to polymer synthesis. It may also be relevant that glucuronic acid forms short side chains in the polysaccharide and is not part of the linear chain (Sutherland, 1970).

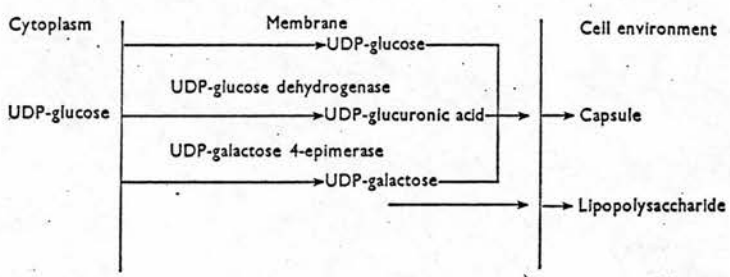
The formation of exopolysaccharides thus appears to be a complex process involving a series of reactions:

(i) formation of intermediates such as UDP-glucose;

(ii) modification of the sugar nucleotides to yield UDP-glucuronic acid;

(iii) sequential transfer of the sugars to a lipid carrier, which is probably an isoprenoid alcohol phosphate;

(iv) polymerization and extrusion into the extracellular environment. Associated with the final stage there must presumably be some mechanism of attachment to the cell surface, as it is known that stable mutants arise yielding exopolysaccharide slime but no discrete capsules (Wilkinson, Duguid & Edmunds, 1954; M. Norval & I. W. Sutherland, unpublished work). The entire process thus



Scheme 1. Postulated structural arrangement for polysaccharide synthesis. UDP-glucose passes through the cell membrane to reach the membrane-bound dehydrogenase and epimerase enzymes. After modification by these enzymes the sugar nucleotides so formed, UDP-glucuronic acid and UDP-galactose, are utilized by the transferases to form precursors for capsule or in the latter case also for lipopolysaccharide. Finally, polymer is excreted from the exterior surface of the membrane.

requires a considerable number of enzymes and a correspondingly large amount of genetic information for the synthesis of structures whose role remains largely unknown and that in the laboratory are not essential for cell viability.

The authors are grateful to Professor E. C. Heath and Dr F. C. Troy for discussions and communication of unpublished results. The technical assistance of Miss I. C. Miller is gratefully acknowledged. Part of this work was financed by a grant from the Science Research Council to Professor J. F. Wilkinson and by a Science Research Council Post-Doctoral Fellowship to M.N.

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glucose' is now known to be a glycerylphosphoryldiglucosyl diglyceride, a new type of phosphoglycolipid (Shaw, N., Smith, P. F. & Verheij, H. M. (1970), *Biochemical Journal* **120**, 439). This structure is based upon a diglucosyl diglyceride containing an additional glycerol phosphate residue on the 6-hydroxyl of one of the glucose residues. Similar phosphoglycolipids have now been isolated from *Streptococcus lactis*, *Leuconostoc mesenteroides* and *Listeria monocytogenes*.

The phosphoglycolipid from *S. lactis* has a similar structure to that isolated from *M. laidlawii*, confirming the observations of Fischer (1970, *Biochemical and Biophysical Research Communications* **41**, 731), who isolated the same lipid from a different strain of *S. lactis*. *L. mesenteroides* and *L. monocytogenes* both contain a glycerylphosphorylgalactosylglucosyl diglyceride. It is significant that the phosphoglycolipids are always structurally related to the diglycosyl diglycerides found in the same organism. The most likely biosynthetic route would be transfer of glycerophosphate from CDP-glycerol to the required diglycosyl diglyceride. Alternatively, phosphatidic acid may be transferred from CDP-diglyceride, giving initially a diacyl glycerylphosphoryldiglycosyl diglyceride which may then lose two acyl residues to give the phosphoglycolipid. Evidence for the occurrence of more highly acylated phosphoglycolipids has now been obtained.

10. Transfer of Monosaccharides to Lipid Intermediates in the Synthesis of Klebsiella Exopolysaccharides. By I. W. SUTHERLAND, MARY NORVAL and I. R. POXTON (*Department of Microbiology, Edinburgh University*)

The synthesis of exopolysaccharide capsules or slime in the genus *Klebsiella* is now known to proceed by stepwise addition of sugars from nucleotide diphosphosugars to a lipid which was shown to be a C_{55} isoprenoid alcohol phosphate (Troy, F. A., Frerman, F. E. & Heath, E. C. (1971), *Journal of Biological Chemistry* **246**, 118; Sutherland, I. W. & Norval, M. (1970), *Biochemical Journal* **120**, 567). The system thus resembles the synthesis of other repeating unit-containing polymers which are found outwith the bacterial cell membrane.

In two different capsular serotypes of *K. aerogenes* the first sugar to be incorporated was glucose (as glucose-1-phosphate). In strain A4 (serotype 8) the addition of glucose is followed by two moles of galactose. Transfer of glucose to the lipid was slightly stimulated by several divalent cations; galactose transfer required either Ca^{2+} or Mg^{2+}. In both cases maximum transfer was obtained when both these ions were present. The optimum pH value for the sugar transferases appears to be between 7·5 and 8·0. Both glucose and galactose transfer increased with increasing membrane protein concentration. Transfer was unaffected by various concentrations of bacitracin but at high chloramphenicol concentrations galactose transfer was partially inhibited. In mutants containing reduced amounts of lipids, bacitracin had an inhibitory effect provided that it was added to growing cells. No inhibition was observed when the membrane preparations were obtained from stationary phase bacteria. The possible exchange of lipid between various polymer-synthesizing systems is therefore proposed.

11. The Biosynthesis of Lipid A in Pseudomonads. By G. O. HUMPHREYS and PAULINE M. MEADOW (*Biochemistry Department, University College London*)

The lipid A of many pseudomonads contains 2- and 3-hydroxy lauric acids (Hancock, I. C., Humphreys, G. O. & Meadow, P. M. (1970), *Biochimica et biophysica acta* **202**, 389). In *Pseudomonas alcaligenes* BRI/2 the sole 12-hydroxy acid is 3-hydroxy lauric acid (Key, B. A., Gray, G. W. & Wilkinson, S. G. (1970), *Biochemical Journal* **120**, 559).

A particulate preparation of BRI/2 incorporated [1-^{14}C]-12:0 into bound 3-OH, 12:0 from either [1-^{14}C]-lauric acid with CoA or [1-^{14}C]-lauryl CoA. The hydroxy acid formed was not extractable by the Folch procedure and was not found in the phosphatidyl ethanolamine fraction. The experiments suggested that 3-OH lauryl CoA was an intermediate in lipid A formation. This ester was synthesized chemically and shown to be a substrate for the particulate system, but the highest activity was obtained with an EDTA preparation of BRI/2 (Robbins, P. W., Wright, A. & Bellows, J. L. (1964), *Proceedings of the National Academy of Sciences of the U.S.A.* **52**, 1302). This preparation was inactive with laurate and lauryl CoA. Attempts to increase its activity by adding possible acceptors were unsuccessful.

Radioactivity bound during incubation of [1-^{14}C]-3-OH 12:0 CoA with the EDTA preparation for 10 min was measured on filter-paper discs after extensive washing with chloroform/methanol

Eur. J. Biochem. 35, 209–215 (1973)

The Production of Enzymes Involved in Exopolysaccharide Synthesis in *Klebsiella aerogenes* Types 1 and 8

Mary NORVAL and Ian W. SUTHERLAND

Department of Microbiology, University of Edinburgh

(Received December 4, 1972/February 2, 1973)

The levels of several enzymes thought to be involved in synthesis of precursors for exopolysaccharide formation in *Klebsiella aerogenes* have been examined in two wild-type strains and in non-mucoid mutants. Enzymes involved in carbohydrate anabolism and catabolism, such as hexokinase and phosphoglucose isomerase, were present at highest levels in the parent strains. Enzymes presumed to be involved in forming precursors for several polysaccharides also had high specific activities, whereas those involved solely in exopolysaccharide synthesis were present at lower levels. Loss of ability to form exopolysaccharide had little effect on the specific activities of the enzymes tested. Some non-mucoid mutants were deficient in one of the enzymes tested or had lowered specific activity, while the specific activities of the other enzymes were unaffected. The only enzymes whose specific activities were significantly increased under conditions favouring exopolysaccharide synthesis were UDP-glucose pyrophosphorylase and GDP-mannose pyrophosphorylase.

All naturally occurring strains of *Klebsiella aerogenes* are mucoid and capsulate. Synthesis of the exopolysaccharide is favoured by certain cultural conditions, including nitrogen or phosphorus deficiency in the presence of excess utilisable carbohydrate, high aeration and lowered incubation temperature [1].

Although it is now possible to postulate many of the enzymes and precursors involved in exopolysaccharide synthesis, little is known about the level of the enzymes or the control of precursor production; both enzymes and precursors may be common to more than one polysaccharide-synthesizing system within the bacterial cell. In some non-capsulate strains of *Diplococcus pneumoniae*, biochemical and genetic defects have been determined [2]. More recently, Ølson *et al.* [3], examined a capsulate strain of *Escherichia coli* K27 and non-mucoid mutants derived from it. The activities of several enzymes involved in synthesis of nucleoside diphosphate sugars were compared in the wild-type and mutant strains. Levels of enzymes (such as hexokinase and phosphoglucomutase) which are involved in a number of pathways, were much higher than those of enzymes *e.g.* UDP-glucose dehydrogenase, required solely for the synthesis of glucuronic acid in the capsule. Lack of capsule production was not always due to failure to synthesise nucleoside diphosphate sugars and it was assumed that some mutations had occurred at later stages in polysaccharide synthesis.

Production of the exopolysaccharide colanic acid, in *Escherichia coli* strain K12, may be controlled through an operon. This was thought to regulate the levels of a number of enzymes involved in synthesis of the nucleoside diphosphate sugars required as precursors [4, 5]. Although *E. coli* K12 has a well-defined genetic system, colanic acid has a complex repeating unit structure [6] and is often only produced in small quantities; yields of enzymes involved are consequently often low. Higher enzyme levels might be encountered in *Klebsiella* strains. Two strains of *K. aerogenes* were chosen: strain A4, which has a simple tetrasaccharide repeating unit containing galactose, glucose and glucuronic acid [7] and strain A1 which secretes a polysaccharide containing fucose, glucose and glucuronic acid [8]. These strains belong to serotypes 8 and 1, respectively. Non-mucoid mutants from both strains were isolated to provide suitable systems for testing the control of enzymes involved in synthesising polysaccharide precursors; in the case of strain A4, UDP-galactose, UDP-glucose and UDP-glucuronic acid and in strain A1, UDP-glucose, UDP-glucuronic acid and GDP-fucose. In both strains UDP-galactose is probably also a precursor of lipopolysaccharide [9]. The transfer of sugars from the sugar nucleotides, such as UDP-glucose to lipid and to exopolysaccharide, was shown using cell-free preparations, confirming their role as precursors in exopolysaccharide synthesis [10].

MATERIALS AND METHODS

BACTERIA AND METHODS OF CULTURE

K. aerogenes strains A1 and A4, described by Dudman and Wilkinson [8], were cultured routinely on nutrient agar at 35 °C. Bacteria were grown on solid or liquid nitrogen-deficient medium [11] and on minimal medium [12]. Lipid cultures were in Erlenmeyer flasks containing half the nominal volume of medium and shaken at 200—300 rev./min in an orbital incubator. Initial isolation of mutants was made on nitrogen-deficient medium or on eosin-methylene blue agar with glucose or other sugar as the carbon source.

MUTAGENESIS

The methods used included: 2-aminopurine, *N*-methyl-*N'*-nitrosoguanidine, acriflavine, ethylmethane sulphonate, $MnCl_2$, nitrite, ultraviolet and γ-irradiation.

PREPARATION OF BACTERIAL EXTRACTS

Cultures (1 l) were grown in nitrogen-deficient or minimal medium at 30 °C for 16 h. The bacteria were harvested, washed in ice-cold saline, resuspended in 10 ml cold 0.1 M Tris-HCl buffer pH 8.0 and disrupted in an ultrasonic generator (M.S.E. Ltd., London) at 20 kHz for 80 s with cooling in ice. Whole cells were removed by centrifugation twice at $2500 \times g$ for 15 min and the supernatant (20—30 mg protein/ml) used as the crude enzyme fraction. The cell-wall-membrane fraction was deposited by centrifugation for 60 min at $30000 \times g$ and resuspended in cold water. Fractions were stored at −20 °C and, when required, thawed slowly.

ASSAY METHODS

Total polysaccharide was determined by a micro-modification of the anthrone technique [13] and protein by the method of Lowry *et al.* [14]. Enzyme assays were performed at room temperature (21 °C), in microcuvettes; in all cases the final volume was 380 µl and the change in absorbance at 340 nm was measured. Glucose-6-phosphate dehydrogenase, hexokinase and phosphoglucomutase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and UDP-glucose dehydrogenase from Sigma Biochemicals (London). Enzyme activity was measured as units/mg protein, where one unit is the amount of enzyme required to transform 1 µmol substrate/min at 21 °C. Any activity found in the control mixtures was subtracted from these values. In all cases, enzymes were assayed at their optimal pH or in systems involving several enzymes, at the optimal pH of the rate-limiting step.

Hexokinase

Hexokinase was assayed by a modification of the method of Slein *et al.* [14a]. The reaction mixture contained 100 µl 1.0 M Tris-HCl buffer pH 7.5, 100 µl 0.1 M cysteine-NaOH pH 8.5, 30 µl 1.0 M $MgCl_2$, 20 µl 0.1 M ATP, 20 µl 0.02 M NADP; 30 µl 0.2 M glucose and 1 µl glucose-6-phosphate dehydrogenase (0.14 unit). The final volume was adjusted to 380 µl with water and the absorbance recorded at 30-s intervals over 30 min.

Phosphoglucomutase

This was assayed by the method of Najjar [15a].

Phosphoglucose Isomerase

The technique of Slein [15] was used. The reaction mixture contained: 100 µl 1 M Tris-HCl buffer pH 7.5, 100 µl 0.1 M cysteine-NaOH, 10 µl 1.0 M $MgCl_2$, 20 µl 0.1 M fructose-6-phosphate, 20 µl 0.02 M NADP and 1 µl glucose-6-phosphate dehydrogenase. In the control, fructose-6-phosphate was replaced by water.

Phosphomannose Isomerase

The method resembled that for phosphoglucose isomerase, the fructose-6-phosphate being replaced by mannose-6-phosphate [15]. The same reaction mixture with glucose-1-phosphate replacing mannose-6-phosphate was used to assay phosphoglucomutase, readings being made for 5 min.

Glucose-6-Phosphate Dehydrogenase

This was determined by a modification of the method of DeMoss [16] using the same reaction mixture as the previous assays with 20 µl 0.1 M glucose-6-phosphate as the substrate. The change in absorbance at 340 nm was measured over 3 min.

UDP-Glucose Pyrophosphorylase

The assay mixture contained: 100 µl 1.0 M Tris-HCl buffer pH 7.5, 5 µl 1.0 M $MgCl_2$, 20 µl 0.02 M NADP, 30 µl 0.05 M sodium pyrophosphate, 20 µl 0.02 M UDP-glucose, 1 µl glucose-6-phosphate dehydrogenase and 2 µl phosphoglucomutase (0.065 unit). Two controls omitting UDP-glucose and sodium pyrophosphate, respectively, were always included [17]. The change in absorbance at 340 nm was recorded over 5 min.

GDP-Mannose Pyrophosphorylase

This was determined according to Munch-Petersen [18] in an assay mixture containing: 100 µl Tris-HCl buffer pH 7.5, 50 µl 0.5 M NaF, 10 µl 0.05 M ADP, 10 µl 0.2 M glucose, 5 µl 1.0 M $MgCl_2$, 20 µl

0.02 M NADP, 20 µl 0.05 M sodium pyrophosphate, 20 µl 0.01 M CDP-mannose, 2 µl hexokinase (0.54 unit) and 1 µl glucose-6-phosphate dehydrogenase. The final volume was adjusted to 380 µl with water, and the absorbance at 340 nm recorded over 10 min.

GDP-Fucose Synthetase

Determinations of this enzyme were made by the method of Ginsburg [19]. The reaction mixture (380 µl) was composed of: 100 µl Tris-HCl buffer pH 8.0, 10 µl 0.02 M NADPH and 20 µl 0.01 M GDP-mannose. The absorbance at 340 nm was measured for 15 min. As for the assay of GDP-mannose pyrophosphorylase, a control lacking GDP-mannose was used.

UDP-Glucose Dehydrogenase

This enzyme was assayed in a reaction mixture [20] containing 50 µl 1.0 M glycine-NaOH buffer pH 8.7, 5 µl 1.0 M $MgCl_2$, 20 µl 0.02 M NAD and 20 µl 0.02 M UDP-glucose. After adjusting the volume to 380 µl with water, the absorbance at 340 nm was recorded over 15 min. A control tube lacked UDP-glucose.

UDP-Galactose-4-Epimerase

Determinations of this enzyme were made in a reaction mixture containing: 50 µl glycine-NaOH buffer pH 8.7, 5 µl 1.0 M $MgCl_2$ 20 µl 0.02 M NAD, 20 µl 0.01 M UDP-galactose and 2 µl UDP-glucose dehydrogenase (1 unit). The final volume was 380 µl and the absorbance at 340 was recorded for 10 min [21].

Crude cell lysates were used throughout as the "enzyme" preparations. Assays were therefore affected by the presence of other enzymes and of endogenous substrates and cofactors. However, the results were sufficiently accurate and reproducible to determine whether a particular enzyme was present and to give a measure of its activity. Most techniques proved satisfactory, but assay of GDP-mannose pyrophosphorylase depended on the presence of two additional enzymes and activities thus determined proved less accurate. In the assay of GDP-fucose synthetase, the reduction of the intermediate GDP-4-oxo-6-deoxy-mannose by NADPH was affected by the non-specific oxidation of the NADPH. Where doubtful results were obtained, the appearance of fucose after hydrolysis of GDP-fucose was determined chromatographically. Similarly, the assay procedure for UDP-glucose dehydrogenase was affected by non-specific reduction of NAD. The reaction may also be inhibited by the product UDP-glucuronic acid. Doubtful results were again checked using chromatography of nucleotide hydrolysates.

RESULTS

Isolation of Non-Mucoid Mutants

Both the *Klebsiella aerogenes* strains A1 (type 1) and A4 (type 8) produced large, raised, shiny colonies of viscous consistency, especially on nitrogen-deficient medium. The capsules formed by strain A1 were very large, approximately 10 µm in diameter, while those formed by strain A4 were smaller. Occasional spontaneous non-mucoid (0) mutants, which had lost the ability to form exopolysaccharide, arose during subculture of both strains. The frequency of O-mutants was increased considerably by the use of mutagens. In an attempt to induce mutations at different chromosomal loci, the various mutagens listed in the Methods were employed. Mutants were thoroughly checked for purity and their pattern of sensitivity to a range of *Klebsiella* phages was compared to that of the wild-type strains. One surprising feature of the mutants was their stability. No mucoid revertants were detected nor could they be induced by further mutagenesis.

During examination of colonies following mutagenesis, two further types of mutation concerned with exopolysaccharide synthesis were noted. One type, which was apparently favoured by aminopurine mutagenesis, was the form which we have designated crenated (CR) mutants. These cultures resembled the wild type on incubation at 35 °C, but were non-mucoid and autoagglutinable at 15—20 °C. Some properties of these conditional mutants have already been described [22]. No mutants of this type were obtained from strain A1. The other type of mutant with altered colonial morphology was a slime-forming variant from strain A1. It was analogous to a similar mutant isolated earlier from *K. aerogenes* type 54 in forming slime of the same chemotype as the parent, but never forming capsules [12]. Such slime-forming (S1) mutants have a similar colonial appearance to the wild-type but can be recognised among well-separated colonies by the greater production of a polysaccharide with increased viscosity.

Determination of Enzyme Levels

The probable enzymes involved in sugar nucleotide formation in strains A1 and A4 are shown in Fig. 1. Although galactose is found in the strain A4 exopolysaccharide, it is also present in the lipopolysaccharides of both strains [9]. Ten of the enzymes thought to be involved in synthesising precursors of the capsular polysaccharides in the two strains were assayed (see Methods).

The enzyme levels in the wild-type strains and in the strain A1 (S1) mutant are shown in Table 1. It proved difficult to work with strain A1, as the large amounts of capsular material present in cell lysates made sedimentation of whole cells extremely

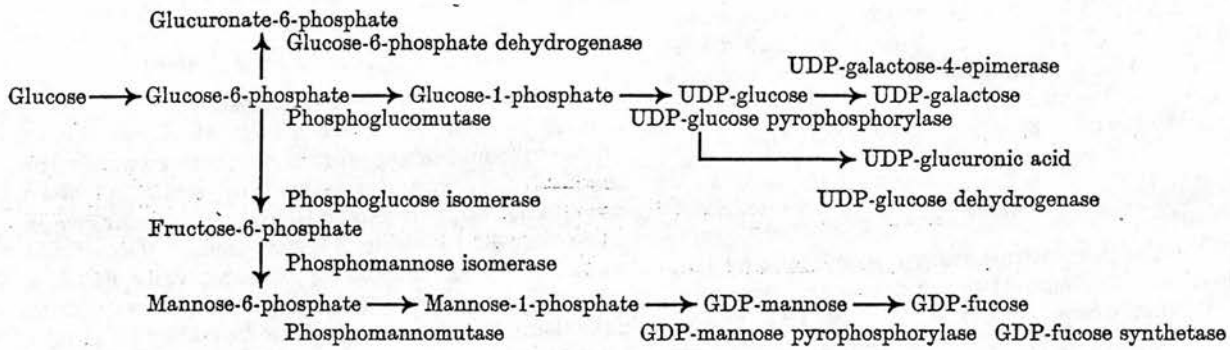

Fig. 1. *Probable pathways leading to the nucleotide sugar precursors of the exopolysaccharides of* K. aerogenes *A1 and A4*

Table 1. *Specific activity of enzymes in cell-free preparations of* K. aerogenes *strains A1 and A4 and the mutant A1 (S1)*
Bacteria were from 16-h cultures grown in nitrogen-deficient medium at 30 °C. The results represent the mean of 6, 2 and 10 separate preparations from A1 (S1), A1 and A4, respectively. The figures in brackets indicate the range of values obtained

Enzyme	Specific activity of		
	Mutant A1 (S1)	Strain A1	Strain A4
	units/mg protein		
Hexokinase	15 (± 3)	43 (± 2)	43 (± 13)
Glucose-6-phosphate dehydrogenase	107 (± 28)	78 (± 10)	22 (± 7)
Phosphoglucomutase	22 (± 4)	—[a]	18 (± 3)
Phosphoglucose isomerase	100 (± 24)	78 (± 10)	19 (± 8
Phosphomannose isomerase	25 (± 5)	—[a]	3.4 (± 0.6)
UDP-glucose pyrophosphorylase	33 (± 10)	—[a]	17 (± 9)
GDP-mannose pyrophosphorylase	4.7 (± 1.3)	—[a]	0.02
GDP-fucose synthetase	0.12 (± 0.08)	0.38 (± 0.1)	0
UDP-glucose dehydrogenase	0.19 (± 0.1)	0.39 (± 0.2)	0.17 (± 0.11)
UDP-galactose-4-epimerase	0.06 (± 0.03)	—[a]	0.9 (± 0.34)

[a] Not tested.

difficult. A high initial absorbance at 340 nm was also present. As preparations from the mutant A1 (S1) were obtained with little contaminating polysaccharide, they were normally used in preference to wild-type material. A noticeable feature was the difference between the enzymes required for normal metabolic function in the cell, such as phosphoglucose isomerase and those thought to be solely involved in polysaccharide synthesis. Although the exopolysaccharide of strain A1 did not contain galactose, the enzyme UDP-galactose-4-epimerase was present, presumably for formation of galactose in lipopolysaccharide. No GDP-fucose synthetase activity was found in strain A4 and negligible GDP-mannose pyrophosphorylase. This was not unexpected as this strain lacks mannose or fucose-containing polymers. The cell-wall-membrane fraction was used as a source of enzymes for cell-free synthesis of exopolysacchraide [10]. It was of interest to determine which enzymic activities were associated with this particulate fraction. Only UDP-glucose dehydrogenase and UDP-galactose-4-epimerase were found in particulate material, while the other enzymes assayed were in the supernatant.

Enzyme Levels in Non-Mucoid Mutants

The mean enzyme levels in a number of independently isolated mutants of strains A1, A1 (S1) and A4 were examined (Table 2). In another four mutants (Table 3) the levels of most enzymes were apparently normal, but the level of a single enzyme in each strain was reduced. Most non-mucoid mutants thus possess normal enzyme levels even for those enzymes thought to be solely involved in the synthesis of precursors for capsular polysaccharide formation.

Two further mutants from strain A4 isolated during attempts to obtain colonies unable to utilise galactose were non-mucoid on minimal medium containing glucose but mucoid on minimal medium supplemented with galactose. One mutant, 039, apparently lacked phosphoglucomutase while other enzymes were present at near normal levels (Table 4). This strain could be grown on two alternative carbon sources, glucose-6-phosphate and glucose-1-phosphate. Using the first of these, good growth was obtained but no exopolysaccharide was formed. In media containing glucose-1-phosphate, growth was poor but some capsular material was formed. The

Table 2. *Enzyme levels of non-mucoid mutants of* K. aerogenes *strains A1, A1 (S1) and A4*
Cells from 16-h cultures in nitrogen-deficient medium grown at 30 °C. The mean value obtained is shown for each enzyme and the variations from this are given in brackets. The number of mutants for each parental type are as follows: mutant A1 (S1), 9; strain A1, 17; strain A4, 16 mutants

Enzyme	Enzyme levels in mutants of		
	Mutant A1 (S1)	Strain A1	Strain A4
	units	units	units
Hexokinase	33 (± 11)	60 (± 20)	25 (± 8)
Glucose-6-phosphate dehydrogenase	62 (± 15)	66 (± 25)	40 (± 10)
Phosphoglucose isomerase	76 (± 20)	63 (± 15)	70 (± 17)
Phosphomannose isomerase	14 (± 5)	20 (± 7)	20 (± 8)
UDP-glucose pyrophosphorylase	38 (± 12)	28 (± 10)	30 (± 10)
GDP-mannose pyrophosphorylase	0.34 (± 0.12)	0.34 (± 0.14)	—[a]
GDP-fucose synthetase	0.12 (± 0.06)	0.17 (± 0.08)	—[a]
UDP-glucose dehydrogenase	0.34 (± 0.20)	0.34 (± 0.20)	0.22 (± 0.11)
UDP-galactose-4-epimerase	—[a]	—[a]	0.90 (± 0.5)

[a] Not tested.

Table 3. *Non-mucoid mutants of* K. aerogenes *A4 and A1 with lowered enzyme levels*
The cell lysates were prepared from 16-h cultures grown in nitrogen-deficient medium at 30 °C

Mutant from parent strain	Mutagen	Enzyme altered	Specific activity of enzyme
			units/mg protein
A4	Aminopurine	UDP-glucose pyrophosphorylase	13
A1	Caffeine	GDP-fucose synthetase	0.05
A1	HNO_2	GDP-mannose pyrophosphorylase	0.1
A1	Acriflavine	UDP-glucose pyrophosphorylase	12

Table 4. *Levels of enzymes in 2 mutants (039,040) of* K. aerogenes *A4 compared with the parent strain*
The cell lysates were prepared from 16-h cultures grown in nitrogen-deficient medium at 30 °C. The figures represent the mean of 3 estimations

Enzyme	Level of enzyme in strain		
	A4	039	040
	units	units	units
Hexokinase	34	25	31
Phosphoglucomutase	19	0.5	19.6
UDP glucose pyrophosphorylase	30	—	0.2
UDP galactose-4-epimerase	0.84	0.60	0.50

second mutant (040) appeared to lack UDP-glucose pyrophosphorylase; the levels of other enzymes resembled those in the parent strain. These were the only mutants which lacked enzymes thought to be involved in the synthesis of sugar nucleotides required for exopolysaccharide formation.

The Effect of Culture Medium on Enzyme Levels

Certain cultural conditions which favour polysaccharide synthesis might be expected to increase levels of enzymes responsible for precursor formation. The enzyme levels of cells of *K. aerogenes* strain A1 (S1) were assayed after growth at 30 °C in a simple synthetic medium in which the only parameters altered were the nitrogen and carbon sources. The basic medium contained 1% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NaCl, 0.1% (w/v) K_2SO_4 and 0.02% (w/v) $MgSO_4$ with the following additions: (a) 1% $(NH_4)_2SO_4$, 1% glucose; (b) 0.03% $(NH_4)_2SO_4$, 1% glucose; (c) 2% casamino acids or (d) 0.03% $(NH_4)_2SO_4$, 1% mannose called media A, B, C and D, respectively.

Cell protein was used as a measure of growth and exopolysaccharide was determined by the anthrone technique. Polysaccharide production/mg cell protein then gave an indication of the relative amount of polysaccharide synthesised and could be correlated with the specific activity of the enzymes (Table 5). Media B and D favoured exopolysaccharide synthesis which was 4—5 times higher than in media A and C. Despite this, the enzyme assays showed little difference between the specific activity of the cells from different media. The largest variations were in the levels of GDP-mannose pyrophosphorylase, GDP-fucose synthetase and UDP-glucose pyrophosphorylase. In media B and D, the activity of GDP-mannose pyrophosphorylase was increased by 100% and that of GDP-fucose synthetase by 180%. Although these enzymes are probably concerned solely with precursors for exopolysaccharide synthesis, the assay techniques showed some variability, with errors in independent estimations of ± 30% and ± 70%, respectively, (Table 1). Activity of UDP-glucose pyrophosphorylase was also higher, but it is also involved in formation of precursors for lipopolysaccharide synthesis.

Table 5. *Enzyme levels and exopolysaccharide production for* K. aerogenes *strain A1 (S1)*
Cells were grown 16 h at 30 °C in different media (see text) prior to lysis. The specific activities for each enzyme are given

Enzyme	Enzyme level in medium			
	A	B	C	D
	units/mg protein			
Hexokinase	9	14	17.9	18.5
Glucose-6-phosphate dehydrogenase	201	140	179	168
Phosphoglucose isomerase	245	174	224	174
Phosphomannose isomerase	25.8	22.5	33.6	26.3
UDP-glucose pyrophosphorylase	25	56	45	84
GDP-mannose pyrophosphorylase	1.7	3.4	1.5	3.4
UDP-glucose dehydrogenase	0.67	0.28	0.6	0.34
GDP-fucose synthetase	0.06	0.17	0.6	0.17
	Exopolysaccharide production in medium			
	A	B	C	D
	µg/mg cells protein			
	30	150	30	120

DISCUSSION

The levels of several enzymes engaged in the synthesis of sugar nucleotide precursors of the exopolysaccharides were assayed in two strains of *Klebsiella aerogenes* and in mutants derived from them. Of 48 mutants, isolated independently with different mutagens, one was defective in phosphoglucomutase. Another lacked UDP-glucose pyrophosphorylase. Four further mutants possessed lowered activity for one of the enzymes tested and the remainder had no detectable defects. This was surprising if it is assumed that the mutagenic agents used caused deletions or alterations to the DNA at different loci. Another unexpected observation was the stability of the mutants, no revertants being obtained. This was also noted for mutants of *Diplococcus pneumoniae*. Three non-mucoid mutants of strain A4 lacked enzymes responsible for transfer of glucose or galactose from their respective uridine diphosphocompounds to a lipid fraction [10]. Genes controlling synthesis and production of sugar nucleotides may be less liable to mutation than other genes involved in polysaccharide production, as are the majority of *rfb* mutants in *Salmonella* strains [23]. These mutants synthesise the "core" lipopolysaccharide structure and the nucleotide sugar precursors for the O-antigenic side chains, but are unable to attach these sugars to the core.

Non-mucoid mutants from strains of bacteria which normally produce extracellular polysaccharide have provided much information about the precursors required for polysaccharide synthesis [24, 25]. However, very little work on the control of the enzymes responsible for sugar nucleotide formation has been reported. The present finding that, in non-mucoid mutants, no uniform lowering or raising of the levels occurs on the enzymes studied, is similar to the results of Olson *et al.* [3]. Mutants obtained from a capsulate strain of *Escherichia coli* also failed to show any correlation between loss of exopolysaccharide formation and the levels of enzymes forming nucleotide sugar precursors. Using a smaller number of mutants, their results did show considerable fluctuations in enzyme levels among the different cultures. No uniform pattern in the enzyme levels could be observed. Unlike this *E. coli* strain or the two *K. aerogenes* strains reported here, the synthesis of colanic acid by *E. coli* K12 shows a different pattern. Investigations by Markovitz and his colleagues [4, 26—29] have shown a complex pattern of control of the synthesis of colanic acid and the enzymes involved. Regulator genes normally repress the synthesis of colanic acid and the level of several enzymes involved in the synthesis of sugar nucleotide precursors. These enzymes included phosphomannose isomerase, UDP-galactose 4-epimerase, UDP-glucose dehydrogenase, GDP-mannose pyrophosphorylase and GDP-fucose synthetase. Derepression, through culture in the presence of *p*-fluorophenylalanine, or through mutation in the regulator genes, resulted in approximately three-fold increases in enzyme levels. Attempts to show similar derepression of capsular polysaccharide synthesis in *K. aerogenes* strains were unsuccessful.

The use of various cultural conditions to cause differences in polysaccharide production did not cause marked differences in the specific activities of the enzyme studied. It is thus possible that in the *K. aerogenes* strains, control occurs at the metabolic level. Accumulation of a particular intermediate may stimulate or repress the activity of some enzyme in the pathway of capsule synthesis. In contrast, the colanic acid system is controlled at the genetic level [4]. Where a nucleotide sugar is required solely

for capsule formation, such as GDP-fucose in strain A1 or UDP-glucuronic acid in both strains, control could be exercised at this point through feed-back inhibition of enzymes of the pathway. Pyrophosphorylases might be the most useful point for such inhibition, as they represent the first stage in commitment of the activated monosaccharide to polymer synthesis. This would account for the results obtained for these enzymes. Several such systems have been demonstrated. They include the inhibition of TDP-glucose pyrophosphorylase by UDP-glucose and TDP-rhamnose [30] and the inhibition of CDP-glucose pyrophosphorylase by CDP-paratose [31]. Some type of negative control might be exercised, by the presence of degradative enzymes breaking down the nucleotide sugar precursors or the cofactors involved in their synthesis. This is seen in the sugar hydrolase studied by Ward and Glaser [32].

The two mutants lacking phosphoglucomutase and UDP-glucose pyrophosphorylase respectively, could both synthesise exopolysaccharide when grown on galactose-containing media indicating a possible similarity to other species of the *Enterobacteriaceae* in galactose metabolism. In *E. coli*, a transport system with high affinity for galactose has been studied [33] and utilisation of galactose through galactokinase and galactose-1-phosphate uridyltransferase has been postulated in *Salmonella* species [34]. Similar mechanisms of uptake and utilisation probably exist in *Klebsiella* species and thus permit incorporation of exogenous galactose into the extracellular polysaccharide of a strain such as A4, which synthesises galactose-containing capsular material. The failure to obtain UDP-galactose-4-epimerase mutants was unexpected as these should have been obtained by the selection procedure used. It is possible, albeit unlikely, that as the lipopolysaccharide of several *Klebsiella* strains contains a very high proportion of galactose [9], such mutants would be essentially devoid of lipopolysaccharide and possess lowered viability.

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M. Norval and I. W. Sutherland
University Department of Microbiology
West Mains Road, Edinburgh, Great Britain, EH9 3JG

Ann. rheum. Dis. (1974) **33**, 453

Cytology of rheumatoid synovial cells in culture

III. Significance of isolates of epithelial cell lines

J. M. K. MACKAY, MARY NORVAL, ALEXIA ROBINSON, DIANA TAIT, HELENA HART, B. P. MARMION, ANN MUIR,* AND W. A. NEILL†
From the Department of Bacteriology, Edinburgh University Medical School, the Department of Haematology, Royal Infirmary of Edinburgh, and the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh†*

Castor, Prince, and Dorstewitz (1961) and Neupert and Sommer (1973) have reported the emergence of continuous cell lines in cell cultures from rheumatoid synovia. Hsu, Pomerat, and Moorhead (1957) noted heteroploid transformation in an epithelial cell derived from a culture of knee joint synovium; the patient was stated to be free from malignant disease, but it was not recorded whether the joint was rheumatoid, arthritic, or healthy. Chessin and Hirschhorn (1961) referred to a permanent cell line which had been established from a normal human synovial tissue.

Ford and Smiley (1973) found aggregations of small round cells in relation to fibroblasts in a synovial culture from a rheumatoid knee; these cells grew in suspension, were not adherent to glass, and synthesized IgG and IgM. They clearly differed from the cells described above and were considered to be B-immunocytes. It might be expected that the ability to grow continuously in culture would be a reflection of the presence of Epstein-Barr virus genome in the cells; however, herpes-like virions were not seen in the cells.

The other cell transformations, as distinct from that described by Ford and Smiley (1973), were glass-adherent cells showing many of the characteristics of the continuous cell lines in common use. During the course of work described in the previous two papers in this series, we have experienced three episodes of such apparent 'spontaneous transformations' in cell cultures from patients with rheumatoid arthritis.

In an investigation of the possible role of viruses in rheumatoid arthritis these observations were initially interesting for evident reasons, namely, the known ability of some viruses to transform cell strains of finite life into lines with indefinite growth potential. However, we were very aware of the results of Gartler (1968), who showed that the majority of continuous cell lines described in the literature as being from different human sources probably had a common origin in the HeLa cell. The most reasonable explanation is laboratory contamination. Investigation along the lines used by Gartler (1968) has shown that the 'epithelial transformation' we have observed in synovial cultures probably represents contamination with Chang 'liver cells'.

Nevertheless, we record our observations for two reasons. First, though these cells were clearly of the Chang (HeLa) type, they differed from the Chang cells we had in our laboratory and may have acquired additional characteristics by virtue of accidental co-cultivation with rheumatoid cells. Secondly, we draw attention again to the dangers of inadvertent contamination with cells, this time in the particular context of cell culture applied to rheumatology.

Materials and methods

Synovial fluid cultures were prepared as previously described (Mackay, Panayi, Neill, Robinson, Smith, Marmion, and Duthie, 1974).

Chang 'liver' cells were obtained from Flow Laboratories (Irvine, Scotland). These cells and the synovial membrane cultures, were prepared or passed by trypsinization; the same basic media were used for all cell cultures but calf serum, rather than mixtures of horse and calf sera, was used for the Chang cells. The general procedures for handling cells followed the methods of Paul (1970).

Chromosome preparations were made by a technique similar to that of McDougall (1970). Species identification by cytotoxicity tests (Greene, Coriell, and Charney, 1964), or by the indirect immunofluorescence reaction, was done with a horse globulin fraction containing antibody to human lymphocytes (a reagent kindly provided by Dr. Keith James, Department of Surgery, University of Edinburgh). Cells were further identified at species level by determination of lactic acid dehydrogenase isoenzyme patterns; the race of origin was checked by electrophoretic

Accepted for publication March 17, 1974.
Requests for reprints to Dr. J. M. K. Mackay, Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG.
This work was supported by a grant from the Nuffield Foundation.

examination for the fast variant of the glucose-6-phosphate dehydrogenase isoenzyme. This isoenzyme is characteristic of many Negro populations and is present in HeLa cells, a line derived from a cervical cancer in a Negress, and is also present in many continuous cell lines, presumably originating from contaminating HeLa cells (Gartler, 1968). Two techniques for isoenzyme assay were used: first the method of de Oca, Macy, and Shannon (1969), but without a vertical starch gel apparatus our results were equivocal, and then the method of Ellis and Alperin (1972) which gave reproducible patterns.

Cells were tested for ability to grow in semisolid agar by the method of MacPherson and Montagnier (1964). For examination in the electron microscope, cells were grown on araldite by the method of Smith, Gray, and MacKay (1969), fixed with 3·5% glutaraldehyde in 0·1 mol/l. sodium cacodylate buffer pH 7·2, and postfixed in 1% osmic acid in the same buffer. They were sectioned with an LKB/ultratome and stained with 1% ethanolic uranyl acetate and Reynolds's lead citrate. Immunofluorescence was by the indirect technique with fluorescein tagged antiglobulin and human sera with antibody or rabbit antisera to various virus antigens. For fluorochrome examination, cells were fixed in Carnoy's fluid and stained with 1/10,000 dilution of acridine orange.

Mycoplasmas were cultured by placing cells in 'sloppy' mycoplasma medium under microaerophilic conditions, subculturing on solid media and identifying the species of isolates by disc inhibition with known antisera.

For estimation of reverse transcriptase activity, cell extracts were prepared after disruption (Coffin and Temin, 1971) and assayed by the method of Temin and Mizutani (1970).

Results

Apparent epithelial transformations were seen on three occasions in a laboratory in the Department of Bacteriology. The first change occurred in a rheumatoid synovial membrane culture (Ref. no. 710025) after 5 serial subcultures; a total of 100 days *in vitro*. When first noticed in a culture held in a Roux flask, large areas of cells of epithelial morphology were seen in association with an intervening monolayer of fibroblasts of the type normally present in synovial membrane cultures prepared in this fashion. When this flask was subcultured an obviously enhanced growth rate was evident. In the two subsequent instances the epithelial 'transformation' was observed in primary synovial fluid cultures as distinct rounded foci, just visible to the naked eye, which enlarged in diameter and in the centres of which dense piling up of cells occurred as incubation was continued. The appearance is illustrated in Fig. 1. These changes were seen after 35 days (Ref. no. 720151) and 16 days (Ref. no. 720301) *in vitro*. When flasks containing these colonies were subcultured they gave rise to even monolayers of epithelial cells resembling those in culture Ref. no. 710025.

Serological characterization by means of cytotoxicity and immunofluorescence tests confirmed that all three cells were of human origin and isoenzyme analysis of lactic acid dehydrogenase confirmed these findings (Table I). At that stage there was some

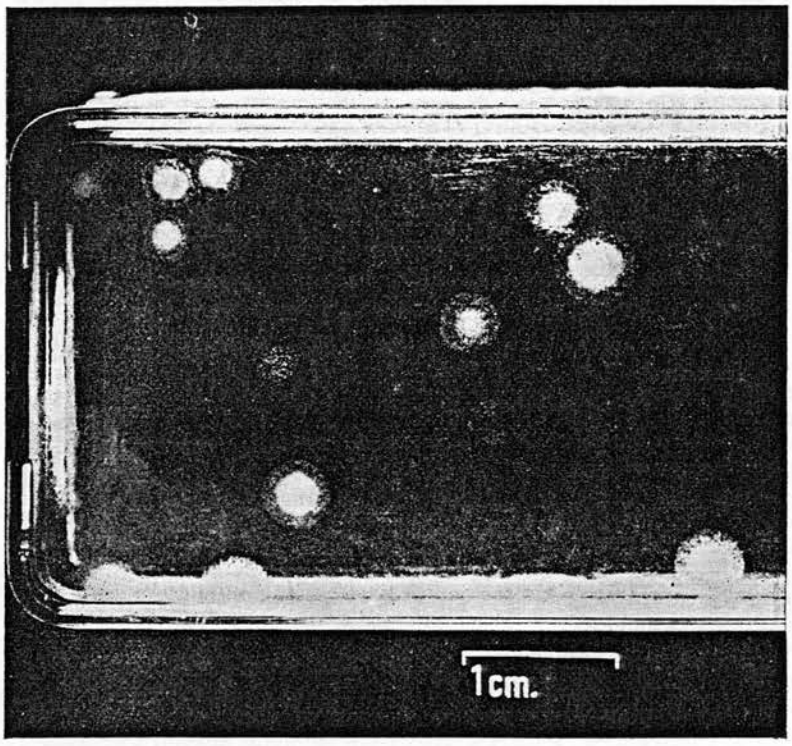

FIG. 1 *Primary synovial fluid culture at 45 days* in vitro, *Ref. no. 720151, showing distinct rounded foci of 'epithelial cells' with dense central areas of thickening*

Table I *Characterization of epithelial cells isolated from rheumatoid synovial cultures by serological and isoenzyme pattern*

Cell isolate or control strain	*Test with antihuman lymphocyte globulin*		*Isoenzyme analysis*	
	Cytotoxicity tests	*Immunofluorescence*	*LDH type*	*G6PD type*
710025	+ve	+ve	Human	A
720151	+ve	+ve	Human	A
720301	+ve	+ve	Human	A
Chang liver cells	+ve	+ve	Human	A
WI-38	+ve	+ve	Human	B
BHK fibroblasts	−ve	−ve	Hamster	Hamster
RK 13	−ve	−ve	Rabbit	Rabbit
710025 RBCs	ND	ND	ND	B
720151 RBCs	ND	ND	ND	B
720301 RBCs	ND	ND	ND	B

ND = Not done.

difficulty in obtaining reproducible glucose-6-phosphate dehydrogenase zymograms and, pending the results of these, certain other features of the cell lines from rheumatoid synovia were examined. These features ultimately included an analysis of the karyotypes of the cells, determination of their ability to form colonies in soft agar, examination of the fine structure of the cells in the electron microscope before and after attempts to induce virus replication by treatment of the cells with bromodeoxyuridine (BUDR) and iododeoxyuridine (IUDR) with dimethylsulphoxide (DMSO) and to assist synthesis of viral proteins by treatment of the cells with actinomycin D.

The problem of the origin of the epithelial cells was finally resolved by determination of their glucose-6-phosphate dehydrogenase isoenzyme patterns on electrophoresis in cellulose acetate strips. A comparison was made between the mobility of G6PD extracted, on the one hand, from the cultured epithelial cells no. 710025, 720151, and 720301, and, on the other hand, from extracts from the erythrocytes of the patients from whom the cells had (ostensibly) come, together with rheumatoid synovial fluid fibroblasts cultured *in vitro*, homogenates of rheumatoid synovial membrane, human embryonic cells, WI-38 human lung fibroblasts, and Chang liver cells. The latter were included because they were the only continuous line cells of human origin present in the laboratory at the time when the apparent 'transformations' had taken place. Some of the results obtained are shown in Table I. It will be seen that the isoenzyme in the 'transformed' cells was in each instance of the fast moving A variant, and quite different from that found in the erythrocytes of the donor. On the other hand, the isoenzyme was the same as that found in the Chang cells. It was concluded that the contamination of the 710025 culture had probably been from the Chang cells and that the other synovial cultures had probably been contaminated with this line. It is of interest in this context that cells 710025, 720151, 720301 were all shown to be contaminated with *Mycoplasma orale*, serotype I; a species known to be present in the Chang cells carried in the department and absent from synovial cultures that had not yielded a 'transformed' epithelial cell.

FURTHER COMPARISONS OF THE EPITHELIAL CELL ISOLATES WITH CHANG CELLS

Although the G6PD isoenzyme analysis eventually clearly showed that cell line 710025 was a contaminant and identified its possible subclones (720151 and 720301), other investigations revealed certain differences between it and the Chang cells.

Karyotype analysis

The frequency distribution of chromosome numbers in cells of 710025 and cells of the Chang cell line is given in Table II. It will be seen that the mean number and standard deviation suggest that the karyotypes of the two populations of cells are different, but both are markedly aneuploid. In addition to these numerical differences, a small submetacentric satellite chromosome was present in 116 out of 200 (58%) metaphase plates of 710025 cells, but was not seen in any of 200 Chang cells (Fig. 2). A similar chromosome was present in 720301 preparations.

GROWTH OF CELLS IN SEMISOLID AGAR

Cell lines 710025, 720151, and Chang were seeded at a density of 10^2, 10^3, and 10^5 cells/Petri dish in semisolid agar and incubated in 5% carbon dioxide in air. Colonies had developed after 7 days' incubation of the plates inoculated with 10^3 cells of 710025 and 720151, and these eventually reached a size of 0·2–0·5 mm diameter by 14 days. There were no colonies in the plates inoculated with 10^5 Chang cells after 14 days' incubation. The colonies in 10^3/710025

Table II *Frequency distribution of chromosome numbers in 100 cells of epithelial isolate 710025 from a patient with rheumatoid arthritis and in 100 cells of the Chang liver continuous cell line*

Cell	*Number of cells with chromosome number of*																*Mean*	*SD*
	<63	*63*	*64*	*65*	*66*	*67*	*68*	*69*	*70*	*71*	*72*	*73*	*74*	*75*	*76*	*>76*		
710025	—	—	1	—	1	4	4	3	13	8	14	31	9	8	2	2	71·84	±2·38
Chang liver	4	7	7	17	25	12	13	3	2	2	2	—	—	—	—	6	66·00	±2·89

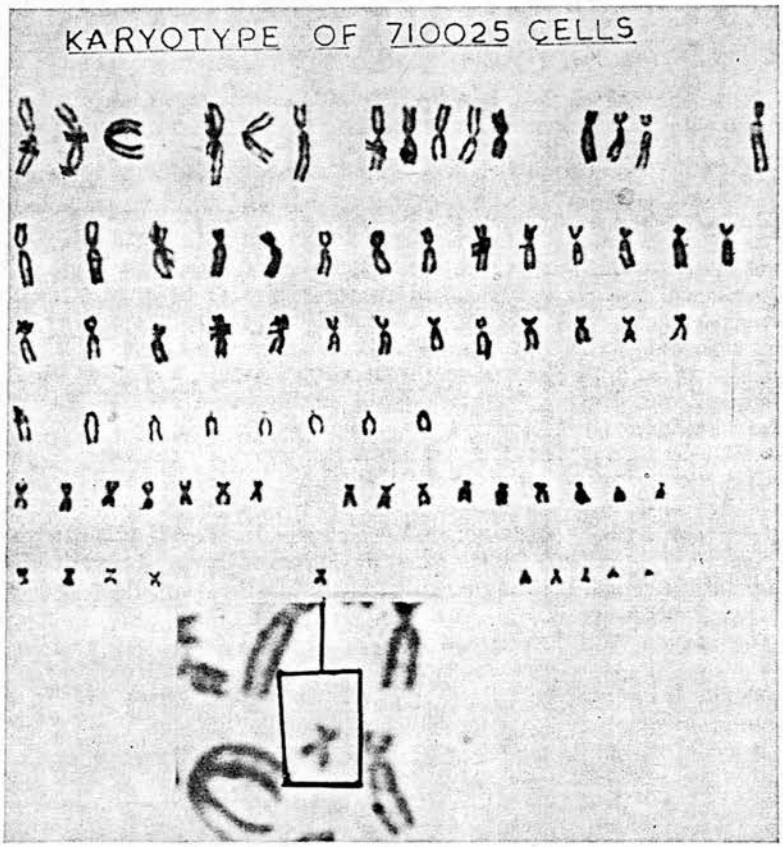

FIG. 2 *Karyotype of 710025 cells illustrating small submetacentric satellite chromosome*

were picked and subcultured in a small Falcon flask and then replated in agar where they again formed colonies.

ULTRASTRUCTURAL STUDIES

Examination of thin sections of all three cell lines showed that approximately 30% of the cells in the culture had surface 'blebs'—that is, distortions of the cytoplasmic membrane with underlying collections of beaded strands of material (Figs 3a and 3b). The beaded appearance may have been produced by the section of tubules, short lengths of which were present in most areas. The outer margins of these structures were electron dense and they varied in diameter from 20 to 25 nm. Collections of this material were also seen within the cell causing severe distortion of the normal cell architecture with displacement of ribosomes, endoplasmic reticulum, and other organelles of the cell (Fig. 3a). These collections were not bounded by membrane and were variable in size. In addition to this material, in a small proportion of the cells there occurred areas of fibrillary material with a more definite outline (Fig. 3a). This sometimes occurred in cells without blebs. The strands were longer and narrower (approximately 15 nm) and arranged in a concentric fashion. Neither type of inclusion was seen in preparations of Chang cells. Because the structures in the cytoplasmic

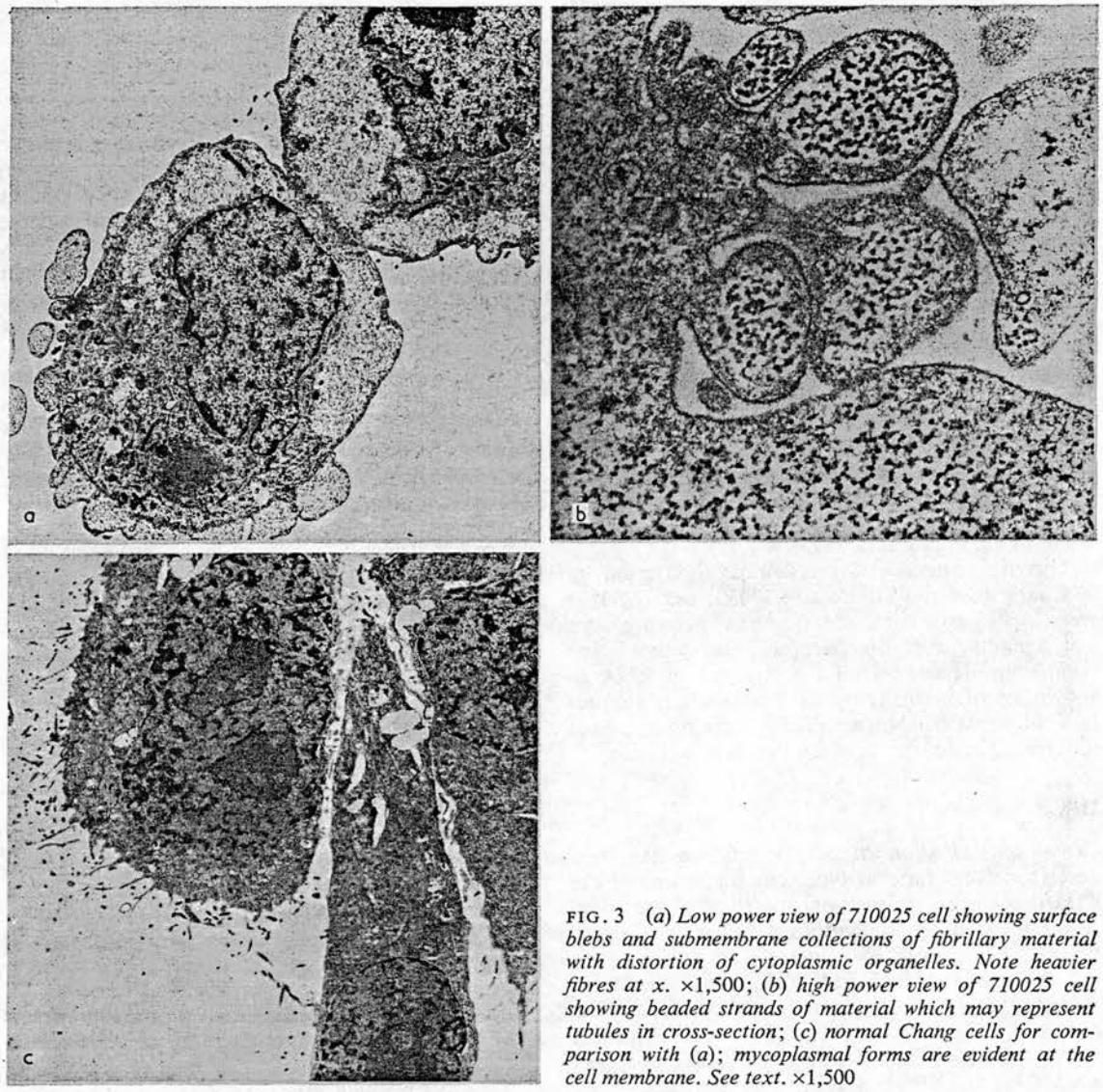

FIG. 3 (*a*) *Low power view of 710025 cell showing surface blebs and submembrane collections of fibrillary material with distortion of cytoplasmic organelles. Note heavier fibres at x.* ×1,500; (*b*) *high power view of 710025 cell showing beaded strands of material which may represent tubules in cross-section*; (*c*) *normal Chang cells for comparison with* (*a*); *mycoplasmal forms are evident at the cell membrane. See text.* ×1,500

blebs resembled the nucleocapsids of certain viruses, notably those of a myxovirus or a paramyxovirus, attempts were made to 'induce' the cells to produce vegetative virus. Treatment with either BUDR or IUDR apparently increased the number of cells with blebs.

Cells were treated with actinomycin D (Sigma) to see if bleb formation and filaments were dependent on a transcription of DNA and also if suppression of cellular mRNA would increase the amount of material. The number of blebs was assessed in preparations stained with acridine orange and by electron microscopy.

Treatment with actinomycin D increased the number of blebs seen in acridine orange preparations and also increased the amount of red cytoplasmic fluorescence. The nucleic acid present was identified as RNA by the use of RNA'ase and DNA'ase treatment.

The treatment with nucleic acid base analogues and with actinomycin D did not lead to the detection, by thin section or negative contrast electron microscopy, of structures resembling complete virus virions, nor did co-cultivation or cell fusion experiments (with lysolecithin as a fusing agent) with these cells and rabbit kidney cells (RK_{13}), human fibroblasts (WI-38), and human glioma transformed by Rous sarcoma virus (118 MG-EH cells) yield virus as judged by cytopathic changes.

In attempts to detect the presence of noncytopathic

or incomplete virions, ^{3}H-uridine was added (5 μCi/ml for 24 hours) to the culture medium of 710025 or 720301 cells previously treated with BUDR (20 μg/ml for 2–3 days). The cells were washed with PBS, harvested, and lysed with distilled water and the cytoplasmic contents fractionated on caesium chloride and sucrose gradients in combination with caesium chloride in the ultracentrifuge (Compans and Choppin, 1967; Yeh and Iwasaki, 1972). Fractions were collected and any fraction forming a peak was examined by negative contrast staining; no structures resembling virions or nucleocapsids were seen.

Incubation at 37°C for 20 mins with 0·1% trypsin removed some labelled material from the cells, but this again had no viral structure either before or after treatment with ether. In addition, lysis of the cells followed by assays for RNA polymerase after sucrose density gradient centrifugation failed to reveal any activity in the appropriate fraction for a paramyxo-like virus (Caliguiri and Tamm, 1970; Compans and Caliguiri, 1973).

There was evidence of reverse transcriptase activity in Chang cells and 710025 and 720301 cells: 720151 were lost due to contamination and therefore were not available for this purpose. The activity was completely dependent on the presence of RNA as shown by the sensitivity of the reaction to pre-incubation with RNA'ase. There were no apparent differences in activity between the three cell lines.

Discussion

Cross-contamination of cells in culture has been described from time to time, but the extent of the hazard is probably less appreciated than the more easily detected contaminations with bacteria, mycoplasmas, or viruses.

In none of the published work on cell lines from human synovial sources is there any evidence of the species of origin of the cells apart from chromosome studies. Chessin and Hirschhorn (1961) observed aneuploidy in the synovial cell line they used. They pointed out that such an appearance could be produced by breakage at the centromeres as was suggested by the high number of telocentric chromosomes. If the karyograms were constructed by re-assembly of the separated chromosome arms then somewhere near the normal human diploid number of 46 could be arrived at. Neupert and Sommer (1973) stressed that their cells diverged in karyotype from other human permanent cell lines. They considered that breakage at the centromeric region may occur during preparation of the chromosome spreads. In a similar fashion to Chessin and Hirschhorn, they constructed karyograms by re-assembly to reach the human diploid karyotype. Neupert and Sommer discarded the simpler explanation that cellular contamination 'specially by L cells' had taken place because continuously propagated cell lines of animal or human origin were not being cultured in their laboratory during this period.

We have been unable to trace the original description of the McCoy cell whose derivation is listed in the Flow Laboratories* catalogue as human synovial, but in correspondence with Dr. Kathleen Cartwright of Flow Laboratories it was pointed out that these cells were still available provided that the customer is fully aware that they are no longer of human origin but have been contaminated with a mouse cell strain. There are no McCoy cells available which are of completely human origin.

The human synovial cell line described by Hsu and others (1957) was lost as a result of yeast contamination and is therefore not available for further analysis. Castor and others' (1961) strain was destroyed in a laboratory accident (C. W. Castor, personal communication, 1973).

Intraspecies contamination may be much more difficult to detect. It has been shown, on the basis of isoenzyme studies, that many of the 'transformed' cell lines of human origin reported between 1952 and 1957 may in fact represent contamination with HeLa cells. 24 human heteroploid cell lines were shown to possess the A variant G6PD isoenzyme found only in some Negroes. Furthermore, the phosphoglucomutase (PGM) phenotypes of 20 of the commonly used cell lines (including Chang liver), were the same as that of the HeLa cell (Gartler, 1968).

Chang liver cells were probably the source of the contamination of the rheumatoid synovial culture yielding the epithelial cell line 710025. After this experience, special precautions were taken to avoid contamination of primary rheumatoid materials, including separate cell culture hoods solely for primary cultures. Despite these precautions, contamination occurred on two further occasions and it is reasonable to suppose either that the source was the Chang cells or the modified line represented by 710025.

It is probable that if the G6PD isoenzyme analyses of the epithelial isolates had worked efficiently at the outset we would have accepted at once the obvious interpretation of cellular contamination and not have examined them further. However, all three rheumatoid cell lines were shown to have other characteristics which were not present in the Chang cells, the probable source of the contamination. It must be admitted that we have no means of ruling out the possibility that the other two rheumatoid lines (720151, 720301) may represent contamination from 710025 cells, and the presence of the marker chromosome in both 710025 and 720301 preparations would suggest this.

The published reports of the isolation of epithelial

* Victoria Park, Heatherhouse Road, Irvine, Scotland KA12 8NB.

cell lines from synovia have sometimes referred to the possibility of cell contamination but have excluded it on the grounds of quantitative karyotypic differences or because no continuous cell lines were being handled in the laboratory. Our experience suggests that the epidemiology of these contaminations is obscure and that all apparent cell transformation should be examined for isoenzyme patterns before it is concluded that transformation of donor's cells has in fact taken place.

Apart from some differences in karyotype and ability to grow in semisolid agar, the most striking feature distinguishing the 710025 epithelial cell line from its presumed parent, the Chang cell, is the presence of blebs and the collections of fibrillary material under the cell membrane and within the cell.

The acquisition of these properties is of interest from several points of view. First, they bear some resemblance to the nucleocapsids of paramyxovirus and certain other RNA viruses. Secondly, they recall the virus-like structures seen in vascular endothelium of the kidney, muscle, skin, and other organs in systemic lupus erythematosis (SLE) (Norton, 1969; Györkey, Min, Sincovics, and Györkey, 1969; Kawano, Miller, and Kimmelstiel, 1969; Schumacher, 1970; Hashimoto and Chandler, 1972). The actual content of the inclusions in 710025 cells is very similar to that seen in the SLE material. The cytoplasmic inclusions are, however, more numerous. Surface blebs are not a feature of SLE tissue in section, but a recent report (Silverman, Chandler, Nieland, and Hashimoto, 1972) mentioned apparent buds of cell membrane containing inclusions which were noted more frequently in SLE cells maintained in culture. The similarity of the tubular inclusions in cultured SLE cells to those in our material is strikingly illustrated in fig. 9 of Hashimoto and Chandler's (1972) paper.

The occurrence of reverse transcriptase activity was not apparently related to the presence of the 'blebs' in 720301 and 710025 cells, but it is nevertheless of interest in view of the recent demonstration by Bykovsky, Miller, Yershov, Ilyin, and Zhdanov (1973) of similar activity in human cancer cell lines. In our case, however, we were unable to find morphological evidence of B-type particles in the cells.

Preliminary investigations suggest that 710025 cells have a surface antigen that differs from those on Chang cells and is stained in membrane immunofluorescence tests with sera from certain patients with rheumatoid arthritis (J. N. McCormick, and others, unpublished data). Both 710025 and Chang cells react with a variety of animal sera containing antibody to paramyxoviruses and other viruses. Adsorption experiments suggest that the antibodies concerned are not directed to viruses but may be part of the T-agglutinin-agglutinogen systems (J. N. McCormick, and others, unpublished data).

There is no explanation at present of the nature of the change produced in Chang cells to give the 710025 cell. This is constantly present on subculture, and is clearly hereditable. A defective viral genome acquired from co-cultivation with the rheumatoid synovial cells is the simplest explanation. It is also possible that actual cell fusion may have occurred since polykaryocytosis is a prominent feature of such synovial cultures.

Summary

On three occasions during the course of culturing cells from synovial membranes and fluids of patients with rheumatoid arthritis the appearance of apparently 'transformed' epithelial cells was observed. When these isolates were characterized by isoenzyme analysis they were found to resemble HeLa cells rather than the cells of the patients from whom the cultures were supposedly derived. Thus, they probably arose as a result of laboratory contamination and attention is drawn to this hazard. The only human cell line in the laboratory at the time the incidents occurred was the Chang liver cell. When the karyological and ultrastructural characteristics of the rheumatoid and Chang cells were examined they were found to differ. In particular, the rheumatoid-associated cells showed accumulations of beaded strands in the cytoplasm and in bleb-like projections of the cytoplasmic membrane. The possibility must be considered that these differences may have arisen as a result of co-cultivation of Chang with rheumatoid cells, albeit accidental.

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DNA polymerase activity in rheumatoid synovial membranes

MARY NORVAL, MARIE M. OGILVIE, AND B. P. MARMION
From the Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG

Norval, M., Ogilvie, M. M., and Marmion, B. P. (1975). *Annals of the Rheumatic Diseases*, **34**, 205–212. **DNA polymerase activity in rheumatoid synovial membranes.** RNase-sensitive DNA polymerase activity was demonstrated in synovial membrane preparations from 23 out of 25 rheumatoid arthritis patients. Control groups consisted of twelve patients with osteoarthrosis, four with secondary osteoarthrosis, and twelve with other conditions. The last group showed no activity, while the results with the other two groups were varied. The properties of the polymerase enzyme, such as its stimulation by synthetic templates and inhibition by actinomycin D, were not consistent with it being associated with an oncogenic virus; it seems to be more like that found in stimulated normal human lymphocytes, described as an RNA-primed DNA-directed DNA polymerase.

The view that an infective agent provides the initiating stimulus for the complex immunological and inflammatory process in the rheumatoid joint is attractive, but little or no definitive evidence has yet been produced to implicate either procaryotes or viruses. Several workers have failed to find either a cytopathic virus in synovial cells or evidence of a substantial, productive, noncytopathic virus infection (reviewed by Marmion, 1975). The remaining possibilities for persistent viral infection of the synovium include a defective, nonproductive, cytoplasmic infection, probably with an RNA virus, accompanied by formation of viral gene products that give a new antigenicity to the host cell. Alternatively, there might be integration of viral DNA into the host cell genome, again with expression of some genes giving rise to host cell modification. Such an integrated viral genome might originate directly from a DNA virus or be copied from an RNA template belonging to an oncorna or leucovirus by virus-coded RNA-dependent DNA polymerase (reverse transcriptase) (Baltimore, 1970; Temin and Mizutani, 1970).

Reverse transcriptase can be measured by the incorporation of ^{3}H-thymidine triphosphate into an acid-insoluble product that can be shown to be an RNA-DNA hybrid sensitive to DNase. The template for the enzyme has a characteristic buoyant density and is sensitive to RNase. The DNA product of the reaction can be extracted and analysed by equilibrium density centrifugation. It is complementary to the template RNA extracted from purified virions, if these are available (Sarngadharan, Sarin, Reitz, and Gallo, 1972).

Accepted for publication October 24, 1974.

These discoveries have opened up new possibilities for the detection of oncorna virus genes in cells. Apart from this approach, general methods of demonstrating an oncorna virus include the visualization of C-type particles in thin sections of cells or tissues in the electron microscope, showing interspecies gs antigen in cells or virions, and the growth of virus or rescue of genetic markers in cell culture.

The present unavailability of an accepted human oncorna virus and lack of knowledge of the cell types that would support its multiplication restrict the different lines of investigation needed to detect and validate oncorna viruses as possible aetiological agents in rheumatoid arthritis (RA). However, morphological studies, examination for gs antigen, and assay for reverse transcriptase might be used to obtain at least preliminary indications of viral activity. Various mammalian cell lines and human neoplasias have recently been investigated in this way (Spiegelman, Kufe, Hehlmann, and Peters, 1973).

Schumacher (1973) and Neumark, Hollos, and Farkas (1973) have reported the presence of C-type particles in thin sections of synovial membrane cells of patients with RA. Palmer and Myers (1973) attempted to show reverse transcriptase activity in a rheumatoid cell line with a questionable result due to the probable presence of a nuclease. However. Spruance, Richards, Ward, and Smith (1974) showed reverse transcriptase activity in cultured rheumatoid synovial cell strains and also found it in normal synovial cell strains.

In general, the demonstration of enzyme activity resembling that of a reverse transcriptase, unsupported by other evidence, cannot be taken as definitive

Table I *Clinical and laboratory details of patients with arthritis*

Clinical classification and case no.	*Age (years)*	*Sex*	*Diagnosis*	*Tests for rheumatoid factor*		
				*SSCT**	*Latex†*	*ANF‡*
Rheumatoid arthritis (RA)						
1	72	F	RA for 6 years	−	..	..
2	67	F	„ 15 „	+	..	..
3	60	F	„ 10 „	+	..	+
4	58	F	„ 1½ „	+	..	..
5	67	F	„ 13 „	−	+	..
6	59	F	„ 4 „	+	..	..
7	61	F	„ 2 „	−	..	..
8	54	F	„ 5 „	+	+	..
9	59	F	„ 6 „	+	..	..
10	60	M	„ 10 „	+	..	..
11	30	F	„ 10 „	−	..	−
12	54	F	„ 5 „	−	..	..
13	36	M	„ 3 „	+	−	+
14	78	F	„ 17 „	..	−	..
15	47	F	„ 1 „	+	+	..
16	66	F	„ 33 „	+	..	..
17	68	F	„ 13 „	+	+	+
18	58	F	„ 10 „	+	−	+
19	64	F	„ 19 „	+	..	..
20	50	M	„ 10 „	+	..	..
21	77	F	„ 14 „	+	..	..
22	56	M	„ 5 „	+	+	−
23	59	F	„ 12 „	−	−	−
24	75	F	„ 25 „	+	..	..
25	24	F	„ 5 „	+	+	−
Osteoarthrosis (OA)						
26	58	F	OA for 6 years	−	−	−
27	52	M	„ 2 „	..	..	..
28	78	F	„ 6 „	−	−	−
29	62	F	„ 7 „	−	−	−
30	61	F	OA now? RA for 2 years	..	−	..
31	70	F	OA for 10 years	..	..	..
32	69	F	„ 5 „	−	−	−
33	77	F	„ 3 „	..	..	..
34	83	M	„ 5 „	..	..	..
35	78	F	„ 2 „	−	..	..
36	74	F	„ 9 „	−	−	−
37	70	F	„ 13 „	−	−	−
'*Secondary*'						
38	43	M	Rotation injury, early OA	−	−	−
39	47	M	OA for 18 years now? RA	−	−	−
40	22	M	Kienböck's disease, secondary OA	−	−	−
41	54	M	OA after injury and arthrodesis	−	−	−
'*Other conditions*'						
42	34	M	Injury 1 year ago	−	−	−
43	25	M	„ 1 „	−	−	−
44	28	M	„ 1 „	..	..	..
45	20	F	„ 2 „	−	−	−
46	20	M	„ 2 „	..	..	..

Table I (*continued*)

Clinical classification and case no.	*Age (years)*	*Sex*	*Diagnosis*	*Tests for rheumatoid factor*		
				*SSCT**	*Latex†*	*ANF‡*
47	20	M	„ 1 „	–	–	–
48	33	M	„ 19 „	–	–	–
49	28	M	„ 2 „	..	..	..
50	38	M	„ 1 „	–	–	–
51	29	M	„ 3 „	–	–	–
52	47	M	„ 2 „	–	–	–
53	57	M	„ 2 „	..	..	..

* SSCT (sensitized sheep cell test) = agglutination of sheep red cells coated with specific rabbit antibody.
† Latex test = agglutination of latex particles coated with IgG globulin.
‡ ANF = antinuclear factor.
+ Indicates a titre of ⩾ 128 on one or more occasions.
.. Indicates that the serum was not available.

evidence of oncorna virus activity. Cell polymerases will synthesize DNA on some synthetic RNA or mixed RNA/DNA templates giving rise to erroneous conclusions. In addition, another DNA polymerase, primed by RNA, has been shown in stimulated, normal human lymphocytes; this can be distinguished from viral reverse transcriptase in several of its properties—in particular its inability to transcribe the heteropolymeric regions of 70S RNA tumour viruses (Bobrow, Smith, Reitz, and Gallo, 1972).

An investigation of the nucleic acid polymerase activity of synovial membranes from patients with RA, degenerative arthritis, and other conditions has been undertaken to explore these problems and potential artefacts in a search for oncorna virus in RA.

Materials and methods

CLINICAL POPULATION (TABLE I)

The synovial membranes from 25 RA patients undergoing synovectomy were used. Controls comprised membranes from 3 groups of patients. The first group of twelve had osteoarthrosis and the second group of four had secondary osteoarthritis. The remaining group, 'other conditions', contained twelve patients requiring arthrotomy after injury, 11 of these having torn menisci.

ENZYME EXTRACTS

Within 2 hours of operation the synovial lining membranes were dissected from the bulk of surrounding fibrous and fatty tissue and cut into very small pieces. The wet weight of the tissue varied from 0·2–5 g, and it was homogenized by hand or with a Potter–Elvehjem homogenizer in a small volume of 0·01 mol/l Tris-HCl buffer pH 8·0, containing 0·001 mol/l EDTA and 0·25 mol/l sucrose. These extracts were frozen and stored at −70°C. The cells were disrupted with Nonidet P-40 according to the method of Coffin and Temin (1971), and the high-speed pellet suspended in a small volume of buffer used in the polymerase assay. Protein content was estimated by the method of Lowry' Rosebrough, Farr, and Randall (1951). The extracts were stored in small aliquots at −70°C and were normally used only once and not refrozen.

POLYMERASE ASSAYS

Assays were performed by the method of Temin and Mizutani (1970) using 0·125 μmol dGTP, 0·125 μmol dATP, 0·125 μmol dCTP, 2·5 μmol phospho(enol)pyruvate, 10 μg pyruvate kinase (320 units/mg), 2 μmol dithiothreitol (all from Sigma), 0·8% Nonidet P-40, 1·25 μmol $MgCl_2$, 2·5 μmol KCl, 2·5 μCi ^{3}H-methyl-TTP (25 Ci/μmol, Amersham), 10, 20, or 40 μl enzyme preparation, and buffer (0·02 mol/l Tris-HCl pH 8·0 containing 0·33 mmol/l EDTA and 1·7 mmol/l 2-mercaptoethanol) to make a total volume of 140 μl. Incubation was carried out at 37°C for a period of between 45 and 120 min. Samples of 30 μl were spotted on filter paper discs at intervals of 15, 20, or 30 min. Filters were fixed in cold 10% trichloroacetic acid (TCA), washed 5 times in cold 10% TCA and then in cold ethanol before being air dried and counted in PPO-toluene scintillator in a Packard Tri-Carb liquid scintillation instrument.

For information on the nature of the primer or template, some samples were pretreated with 10 μg pancreatic ribonuclease (100 units/mg, Sigma) at 0°C for 10 min before adding the assay constituents. Ribonuclease had previously been heated at 70°C for 10 min to destroy any deoxyribonuclease activity.

The artificial templates/primers poly-deoxy-adenylic-deoxy-thymidylic acid (poly-d(A-T)) and polyadenylic acid decathymidylic acid (poly-A.dT_{10}) were used at concentrations of 0·07 OD_{260} units/ml (Boehringer Corp.).

The effect of actinomycin D was found by the addition of 50 μg/ml (Sigma) dissolved in polymerase buffer at the start of the reaction.

PRODUCT ANALYSIS

Standard reaction mixtures as outlined above were scaled up to 0·6 ml and were incubated at 37°C for 20 min. The products were extracted with phenol-cresol and analysed on Cs_2SO_4 density gradients (Sarngadharan and others,

Table II *Polymerase activity of synovial membrane cells, expressed as increase in cpm incorporated into acid insoluble material in 30 min/μg protein*

Case no.	*Total polymerase activity (A)*	*Polymerase activity in presence of RNase (B)*	*RNase-sensitive DNA polymerase (A-B)*
RA group			
1	4·5	0·7	3·8
2	2·1	0·7	1·4
3	1·4	0·4	1·0
4	2·3	0	2·3
5	0.7	0.5	0.2
6	1·5	1·5	0
7	0·9	0·4	0·5
8	2·7	1·4	1·3
9	0	—	0
10	7·7	3·9	3·8
11	3·4	1·9	1·5
12	0·4	0·2	0·2
13	2·2	1·3	0·9
14	2·6	1·1	1·5
15	3·2	1·4	1·8
16	3·6	1·3	2·3
17	0·4	0·1	0·3
18	2·0	0·5	1·5
19	1·8	0·5	1·3
20	5·1	1·7	3·4
21	3·0	0·4	2·6
22	8·0	0	8·0
23	3·0	1·0	2·0
24	0·8	0·4	0·4
25	0·8	0·3	0·5
OA group			
26	1·1	0·8	0·3
27	0·5	0·5	0
28	0	—	0
29	1·9	1·1	0·7
30	0·5	0·5	0
31	3·6	1·4	2·2
32	0	—	0
33	0	—	0
34	0·7	0·4	0·3
35	0	—	0
36	0	—	0
37	0·6	0·4	0·2
'Secondary' group			
38	2·0	0·8	1·2
39	6·0	3·3	2·7
40	1·1	0·9	0·2
41	0	—	0
'Other conditions'			
42	0	—	0
43	0·6	0·6	0
44	0	—	0
45	0	—	0
46	0	—	0
47	0	—	0
48	0	—	0
49	0	—	0
50	0	—	0
51	0	—	0
52	0	—	0
53	0	—	0

1972) or glycerol gradients (Gulati, Axel, and Spiegelman, 1972).

Results

The crude enzyme extracts prepared by high-speed centrifugation were assayed for the presence of endogenous RNase-sensitive DNA polymerase. Table II shows the result of the assays done, first without ribonuclease, then after preincubation with this enzyme. The activities of the membranes are expressed as an increase in acid-precipitable counts per minute over the first 30 min of the incubation per mg protein. Normally the mean value from two enzyme concentrations was recorded. With some membranes the incorporation of labelled nucleic acid precursors into acid-insoluble material was not a linear function of time after about 30 min. Fig. 1 shows the assay of membrane of Case 16, an RA patient. Some membranes, particularly from the patients without arthritis, showed no increase in acid-precipitable counts over the normal incubation period. In these cases, 40 μl samples of enzyme extract were used and the incubation continued over 2 hours, still with no demonstrable activity.

It may be seen from Table II that there is some polymerase activity not dependent on RNA as template. This is presumably due to the presence of some cellular DNA polymerases in the crude enzyme preparation. In the final column in Table II, the polymerase activity dependent on RNA is obtained by subtracting the activity after preincubation with RNase from that without RNase treatment. This is taken as being endogenous RNase-sensitive DNA polymerase activity.

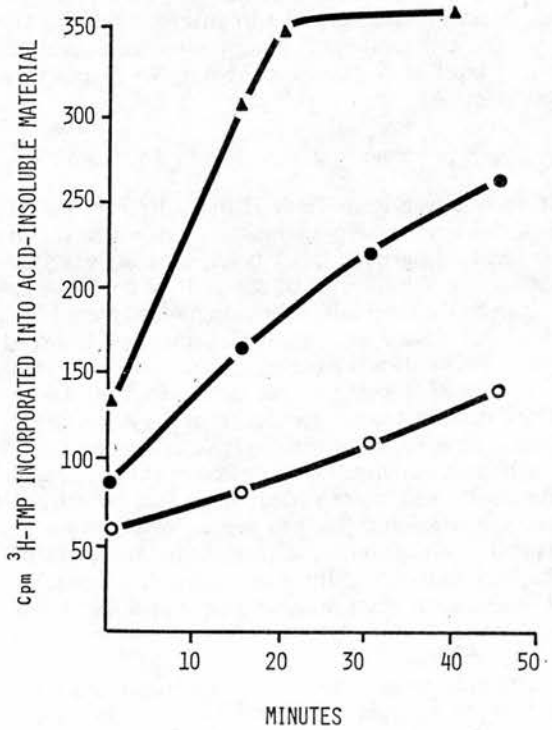

FIG 1 *Endogenous DNA polymerase activity of RA synovial membrane from Case 16. Assay conditions as in Methods, using 20 μl crude enzyme preparation ●, 40 μl ▲, and 20 μl after preincubation with RNase ○*

The presence of this activity was of interest as a possible marker of oncorna virus, particularly as there was a clear-cut difference between the four groups. The mean activity for the RA group was 1·7, for osteoarthrosis 0·3, for 'secondary' 1·0, and for 'other conditions' 0. However, there are obvious differences between RA synovial membrane on the one hand and the controls on the other. The former is characterized by massive proliferation of lining cells and extensive infiltration with cells of the lymphocyte series. As these cells have an RNA-primed DNA polymerase (Bobrow and others, 1972), it was necessary to determine whether the activity in the RA membranes (in particular) was of this type or was a true viral RNA-dependent DNA polymerase.

It is known that crude preparations of viral polymerases prefer hybrid synthetic template/primers such as poly-A.d(T)$_{10}$ to double-stranded DNA synthetic templates such as poly-d(A-T), while this is not true of the cellular polymerases (Goodman and Spiegelman, 1971). The activity of some membranes was therefore determined in the presence of these two synthetic templates and, as shown in Table III, poly-d(A-T) at least doubled DNA synthesis, whereas poly-A.d(T)$_{10}$ had no effect.

High concentrations of actinomycin D inhibit DNA-directed DNA synthesis but do not affect the first step of viral reverse transcriptase activity, which is the synthesis of an RNA-DNA hybrid (McDonnell, Quintrell, Garapin, Fanshier, Levinson, and Bishop,

Table III *Effect of artificial templates on polymerase activity of synovial membranes, expressed as a ratio of endogenous activity to activity in the presence of poly-d(A-T) and poly-A.d(T)$_{10}$*

Case no.	*Endogenous*	*Poly-d(A-T)*	*Poly-A.d(T)$_{10}$*
RA group			
12	1	4·7	1·2
19	1	4·2	1·1
20	1	1·2	0·7
21	1	3·2	1
22	1	2·0	1·2
OA group			
37	1	3·5	1·2
'Secondary' group			
39	1	—	0·8

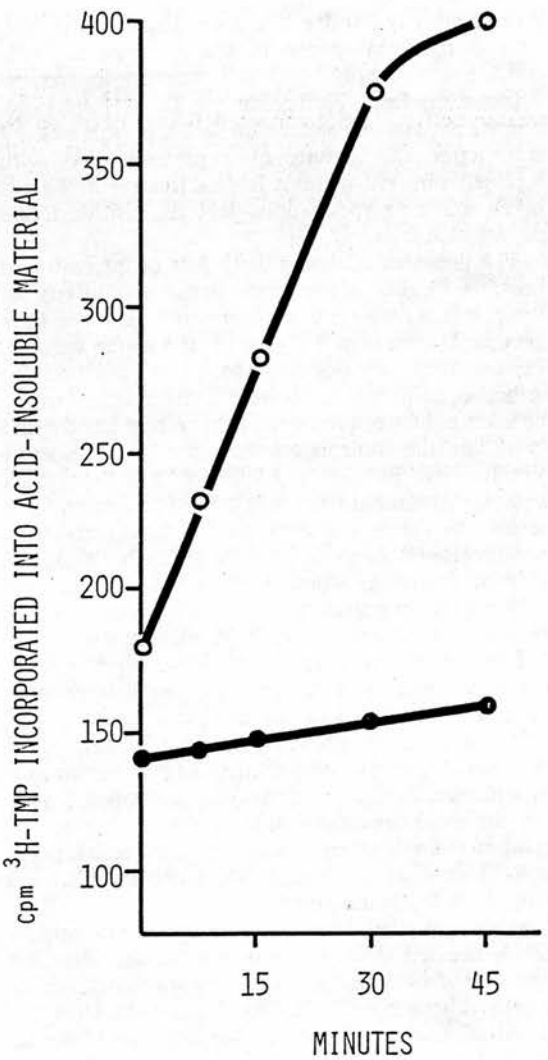

FIG. 2 *Effect of actinomycin D on endogenous DNA polymerase activity of synovial membrane of Case 39 from 'secondary' group. Actinomycin D (50 µg/ml dissolved in polymerase buffer) was added to assay mixture ● or polymerase buffer alone ○*

1970). Actinomycin D was therefore added to the assay mixture of several membranes and samples taken at 7, 15, 30, and 45 min. Even at 7 min there was marked inhibition of incorporation into acid-insoluble product and this is illustrated for one membrane (Case 39) from the 'secondary' group in Fig. 2. Table IV shows the inhibition in the 11 membranes tested.

Finally, an attempt was made to analyse the endogenous product of the polymerase system of Cs_2SO_4 and glycerol gradients. It proved difficult to obtain a membrane preparation of sufficient size and activity to do this satisfactorily. However, with the few membranes tried there was never any suggestion of the formation of an intermediate RNA-DNA hybrid. This should band at a density of about 1·66 g/ml on Cs_2SO_4 equilibrium density centrifugation (Sarngadharan and others, 1972) and have sedimentation coefficient of 70S on a glycerol gradient (Gulati and others, 1972). All the radioactivity banded in the DNA region, that is with density around 1·41 on Cs_2SO_4 equilibrium density centrifugation and near the top of the glycerol gradient.

As the above results were not indicative of a viral RNA-dependent DNA polymerase, it was thought necessary to check the assay conditions using a known positive control. Use was made of a human glioma cell line transformed with Rous sarcoma virus, 118 MG-EH (Pontén and Macintyre, 1968) (kindly provided by Dr. Jan Pontén). A pellet was prepared from cell lysates as described for the synovial membrane cells. In this case there was stimulation of incorporation into acid insoluble material by at least fifteenfold in the presence of poly-d(A-T), and in addition poly-A.$d(T)_{10}$ stimulated the activity by at least threefold. There was still incorporation of ^{3}H-TMP into acid-insoluble product over the first part of the incubation period in the presence of actinomycin D, although it eventually caused about 40% inhibition of activity. As with the membrane preparations, it was difficult to obtain sufficient product to analyse it on Cs_2SO_4 and glycerol gradients, though evidence suggested that a labelled intermediate RNA-DNA hybrid was being formed.

Discussion

It may be seen from Table II that 23 out of the 25 synovial membrane preparations from patients with RA had endogenous DNA polymerase activity. Although the dependence of the activity on RNA as shown by the sensitivity of the reaction to preincubation with RNase was variable, in most specimens it was substantial and in marked contrast to membranes from osteoarthrotic patients or traumatized joints. Thus, none of the membranes from the nonarthritic group showed any endogenous RNase-sensitive DNA polymerase activity. Of the osteoarthrotic patients the results were more varied; seven had no activity and the remaining five had some. In the group of patients with secondary osteoarthritis, three out of the four showed activity. The residual activity, after RNase treatment, is presumably due to cellular DNA-dependent DNA polymerases present in the crude enzyme preparations.

The difference between RA membranes and controls could be explained in various ways. One of the characteristic features of the former is the proliferation of the synovial membrane and it is obviously difficult to obtain adequate control material in terms of cellular composition for comparative observations.

Table IV *Effect of actinomycin D (Act D) on polymerase activity of synovial membranes, expressed as increase in cpm ^{3}H-TMP incorporated into acid-insoluble material after 30-min incubation*

Case no.	*Polymerase activity*	*Polymerase activity in presence of Act D*	*Activity with Act D* % *Activity without Act D*
RA group			
12	48	8	16·7
19	290	25	8·6
20	680	53	7·5
21	200	15	7·5
22	230	13	5·7
25	35	0	0
OA group			
31	42	8	19·0
34	40	4	10·0
37	70	0	0
'Secondary' group			
38	33	5	15·0
39	195	12	6·2

Membranes from patients in 3 other categories have been used: those with osteoarthrosis, those requiring arthrotomy following injury, and those from a small group with secondary osteoarthritis. These control membranes, particularly those from patients without arthritis, were quite different in morphology and in size from the rheumatoid membranes. It should be noted that, in addition to the variation in morphology of the synovial membranes in the different groups, they were not matched for age and sex; for instance, the RA patients tended to be female with average age of 58 years, whereas the nonarthritic patients tended to be male with average age of 32.

Apart from proliferation of synovial lining cells, RA membranes are frequently infiltrated with large numbers of mononuclear cells of the lymphoid series, usually in excess of any inflammatory exudate found in degenerative or traumatic arthritis.

It is therefore necessary to consider whether the RNase-sensitive DNA polymerase activity found in RA synovial membranes is the product of an oncorna virus or if it is associated with the presence of a RNA-primed DNA polymerase of the type found in stimulated normal human lymphocytes (Bobrow and others, 1972). Several characteristics of the membrane enzyme suggest that, in fact, it is more likely to be from lymphocytes than virus. Thus, activity of the enzyme was not stimulated by poly-A.d$(T)_{10}$, which is reported to increase DNA synthesis by viral reverse transcriptase, and actinomycin D inhibited the enzyme. These findings are the reverse of those described for viral enzyme and in line with our own control experiments with extracts from a human glioma cell line (118 MG-EH) containing the Rous sarcoma virus genome. Finally, although it was difficult to obtain satisfactory quantities of the nucleic acid product of the polymerase reactions given the low levels of enzyme activity in the membranes (compared with those in the virions) ultracentrifugal analysis of phenol extracts of the synthesized material on glycerol and caesium sulphate gradients did not reveal a RNA-DNA hybrid, only DNA of small sedimentation constants.

We were unable, therefore, to find evidence of the presence of an oncorna virus in RA synovial membranes in terms of RNA-dependent DNA polymerase. This observation agrees with limited attempts by Grayzel (1973), who used cultured rheumatoid synovial cells and was unable to show incorporation of isotopically labelled uridine into particles of characteristic buoyant density on sucrose gradients; a marker for liberation of virus from cells.

It is not clear, however, from the literature whether failure to show reverse transcriptase is equable with the absence of an oncorna virus genome, or part of it, from a cell. The 118 MG-EH cell line and a hamster cell line, HT-1, transformed by murine sarcoma virus (Karpas, Cawley, Tuckerman, Flemans, and Hayhoe, 1971) both had viral reverse transcriptase. Although the former contains gs antigen, it is apparently free from virus particles (Macintyre, Grimes, and Vatter, 1969), and neither virions nor gs antigen have been shown in the latter (Karpas and others, 1971). On the other hand, Holder, Robey, and Vande Woude (1974) have shown with HBT-3 cells (supposedly a human breast tumour cell but with HeLa cell chromosomes) that reverse transcriptase activity is low except in cells treated with hormones and inducers; so perhaps the viral genes for the enzyme may not always be expressed and further experiments with rheumatoid synovial membranes and fibroblasts are necessary before final conclusions can be drawn.

This work was supported by a grant from the Nuffield Foundation. The HT-1 cells were kindly provided by Dr. A. Karpas. We wish to acknowledge the help of the consultant surgeons at the Princess Margaret Rose Hospital, Edinburgh, for providing synovial membrane specimens. We thank the staff of the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh, for providing laboratory details of patients' sera.

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Attempts to identify viruses in rheumatoid synovial cells

MARY NORVAL AND B. P. MARMION
From the Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG

Norval, M., and Marmion, B. P. (1976). *Annals of the Rheumatic Diseases*, **35**, 106–113.
Attempts to identify viruses in rheumatoid synovial cells. Synovial fibroblast cell strains derived from the synovial membranes of 7 patients with rheumatoid arthritis were examined for the presence of viruses, in particular leucoviruses. Seven similar synovial strains derived from patients with other arthritic conditions were used as a control group. Evidence of the presence of a virus or a viral genome was looked for by several methods of induction followed by ^{3}H-uridine labelling of the cultures. In addition, the culture supernatant, after induction and after the synovial strains had been co-cultivated with a variety of cell lines from several species, was assayed for the presence of viral RNA-dependent DNA polymerase activity. The DNA-polymerase activity of the synovial cells themselves was also determined. No evidence was found by any of these techniques to indicate the presence of virus or viral information within the synovial fibroblasts.

Some simple methods have been developed to grow synovial cells from rheumatoid and nonrheumatoid synovial membranes (Castor and Fries, 1961). These cells can be passed by trypsinization and propagated through at least 15 subcultures. Various workers have looked at these cultures to find differences between the rheumatoid and nonrheumatoid cells which could indicate some abnormality in the rheumatoid cells (Bartfield, 1965; Smith, 1971; Phillips, 1971; Smith and Hamerman, 1969) and in particular some evidence of viral infection.

Generally the rheumatoid cultures grew more quickly but could not be subcultured as many times as the nonrheumatoid cultures. At the ultrastructural level no differences could be detected (Wynne-Roberts and Castor, 1972). No nuclear bodies were seen and no cytopathic effect on prolonged co-cultivation. However, there were various biochemical differences in the cultures, such as increased hyaluronic acid content of rheumatoid cells and increased acid phosphatase activity. It is known that some viruses can interact with the host cell to alter some cellular metabolic activities; for example, avian sarcoma viruses induce increased hyaluronic acid production in infected chick embryo fibroblasts (Ishimoto, Temin, and Strominger, 1966).

Using conventional virological techniques on rheumatoid synovial fibroblasts, no virus has been isolated after co-cultivation or by Sendai-induced cell fusion, nor did the cells show haemadsorption or haemagglutinins and there was no interference with VSV or ECHO-11 replication (Phillips, 1971). Ford and Oh (1965) reported no virus in the culture supernatant after prolonged incubation and no difference in NDV susceptibility. Smith and Hamerman (1969) also failed to isolate a virus from these cells although they showed a resistance to infection with rubella and NDV in the case of the rheumatoid cells. This has been attributed to the increased hyaluronic acid content of the rheumatoid cells compared to the nonrheumatoid (Clarris, Fraser, and Rodda, 1974; Patterson and others, 1975). However, it has also been shown that two means of transferring this resistance to rubella are by intra-articular injection into rabbit joints whereby the rabbit synovial membrane cells become resistant 2 to 6 months later (Smith and others, 1974), and by Sendai-virus induced cell fusion of these cells with normal rabbit synovial cells (Smith and Hamerman, 1974). No virus was released from these cultures.

It is possible that, should a virus be concerned in the aetiology of rheumatoid arthritis, the infection may be a nonlytic one and thus would not be detectable by normal techniques. There are several possibilities for a persistent viral infection of this kind. The virus might be produced in small numbers and be noncytopathic. Alternatively there might be a defective nonproductive cytoplasmic infection with

Accepted for publication November 1, 1975.
Correspondence to Dr. Mary Norval.

formation of new viral-coded antigens on the cell surface. As a third possibility, the viral genome might be integrated into the host cell genome with some accompanying changes in the cell. In the case of DNA viruses this integration can occur directly; with some RNA viruses, as shown with leucoviruses, a DNA copy of the RNA is made first using RNA-dependent DNA polymerase (reverse transcriptase) before integration occurs. Recently some of the techniques used in leucovirus work have been applied to synovial membranes and strains to ascertain if a virus of this type is present (Norval, Ogilvie, and Marmion, 1975; Spruance and others, 1975).

Person, Sharp, and Rawls (1973) have studied cell cultures from synovial fluids and membranes after co-cultivation with Vero cells and after induction with 5-iododeoxyuridine (IUDR.) ^{3}H-uridine or ^{3}H-thymidine was added and the culture supernatant subsequently examined by sucrose and caesium chloride density gradient centrifugation to isolate labelled particles. No virus was found. Grayzel (1973) has also described the result of adding ^{3}H-uridine to the medium of cultured rheumatoid cells with and without pretreatment with 5-bromodeoxyuridine (BUDR) and actinomycin D. There was no evidence of virus particles being released from the cells or of viral-specific RNA being synthesized within the cells.

In this paper we describe the properties of a collection of synovial fibroblast cell strains from rheumatoid arthritis patients. Strains from other arthritic conditions were included as controls. Evidence of the presence of a virus or viral information was sought by several methods of induction followed by ^{3}H-uridine labelling and by assaying the culture supernatant for reverse transcriptase activity. In addition, various techniques of co-cultivation were tried in an attempt to rescue a defective virus. The DNA polymerase activity of the synovial cells was also assayed.

Materials and methods

SOURCE OF CELL STRAINS

The lining cells from synovial membranes obtained at synovectomy were dissected out and cut into small pieces. These were trypsinized (0·25% trypsin in Dulbecco's solution) overnight at 37°C and the free cells removed into Eagle's complete medium containing 10% fetal calf serum. Monolayers of fibroblasts were passed by trypsinization. In addition, 4 strains grown out from explants of synovial tissue were received from Dr. D. Hamerman (A, B, C, and D). The synovial fibroblast strains used and the diagnosis of the patients from whom the specimens came are shown in Table I. The cell strains were tested at intervals for the presence of mycoplasmas as described previously (Mackay and others, 1974).

Table I *Source of synovial fibroblast strains*

Strain	*Diagnosis*
A	RA
B	RA
C	Congenital hip displasia
D	Osteoarthrosis
E	RA
F	RA
G	Osteoarthrosis
H	RA
I	Polyarthritis (non-RA)
J	RA
K	Osteoarthrosis
L	Osteoarthrosis and Paget's disease
M	RA
N	Osteoarthrosis

ULTRASTRUCTURE

Cells were harvested using glass beads and prepared for electron microscopy. In addition, cells were regrown for 3 days after storage in liquid nitrogen before being harvested for electron microscopy. The special glycogen stain of De Bruijin (1973) and Schaff, Barry, and Grimley (1973) was used on cell strain B. The blocks were sectioned on an LKB Ultratome 11 and the sections viewed on a Hitachi HU11A electron microscope.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME

The method of Ellis and Alperin (1972) was used.

ATTEMPTS TO INDUCE VIRUS PRODUCTION

(A) Induction followed by ^{3}H-uridine labelling

Some of the cell strains were incubated in the presence of BUDR (20 μg/ml) for 3 days (Lowry and others, 1971), IUDR (20 μg/ml) for 3 days followed by dimethylsulphoxide (DMSO) (2%) for 4 days (Stewart and others, 1972), cycloheximide (10 μg/ml) for 16 hours (Aaronson and Dunn, 1974), or with mitomycin C (1 μg/ml) for 18 hours in the dark (Weiss and others, 1971).

The medium was replaced with the minimal volume of Eagle's medium containing 1% fetal calf serum and (5-^{3}H) uridine (2·5 μCi/ml, Amersham) and incubation continued for a further 24 hours. Ammonium sulphate precipitation of the culture supernatant was carried out according to the method of Grayzel (1973). The precipitate was dissolved in 1 ml 0·01 mol/l TRIS-HCl, 1 mmol/l EDTA pH 7·1, layered on the top of a linear sucrose gradient of 20 to 70% (w/v) sucrose in buffer, and spun at 24000 r.p.m. for 18 hours at 4°C in a SW 40 rotor. Fractions of about 0·5 ml were collected from the bottom and their refractive index measured. 1 ml cold 10% trichloracetic acid and 50 μl 0·2% bovine serum albumin were added to each fraction and after at least 20 minutes at 4°C the precipitate was collected on glass fibre filter paper discs (Whatman GF/A), washed extensively with cold 10% TCA, followed by cold ethanol. They were air-dried and counted in PPO-toluene scintillator in a Packard Tri-Carb liquid scintillation instrument.

(B) Induction with testosterone and IUDR

The procedure of Holder, Robey, and Vande Woude (1974) was used. The cultures were incubated in the presence of testosterone acetate (5 μg/ml) for 8 weeks, being split as necessary, usually once every 2 weeks. The

medium was changed weekly. The supernatant was collected at 2-week intervals and assayed for reverse transcriptase activity using the method of Lieber and others (1973). Finally IUDR (100 μg/ml) was added for 1 day before the final supernatant sample was collected.

CO-CULTIVATION

(*A*) *XC test*
The procedure of Rowe, Pugh, and Hartley (1970) was used.

(*B*) *With various cell lines*
The procedure outlined by Benveniste and others (1974) for inducing C-type virus from baboon placenta was used. The cell lines tried were LLC-MK_2 (monkey kidney cells), D-17 (dog fibrosarcoma cells), XC (rat tumour cells transformed by Prague strain of Rous sarcoma virus), 118 MG-EH (human glioma cells transformed by murine sarcoma virus), HT-1 (hamster cells transformed by murine sarcoma virus), and human fetal skin fibroblasts. 6×10^5 cells were seeded in a medical flat, incubated overnight, and induced with 30 μg/ml IUDR for 24 hours. 1×10^5 cells of the various lines were added and incubation continued for 8 weeks. Culture medium was changed weekly and the cultures were split at fortnightly intervals. The supernatant was assayed every 2 weeks for the presence of reverse transcriptase activity using the method of Lieber and others (1973).

TRANSFECTION
DNA was extracted from the cells using the method of Karpas and Milstein (1973) and Karpas and Tuckerman (1974). Human fetal skin fibroblasts were subsequently exposed to it. The cells were cultured in Eagle's medium containing 10% fetal calf serum and split at about 10-day intervals.

DNA POLYMERASE ACTIVITY

(*A*) *Culture supernatant*
Induction with BUDR or testosterone of the synovial cell strains was carried out as indicated previously, and 20 ml culture fluid prepared and assayed according to the method of Lieber and others (1973).

(*B*) *Cells*
Synovial fibroblasts, grown in 4 or 5 Roux flasks, were washed in medium and prepared as outlined by Coffin and Temin (1971). Protein content was estimated by the method of Lowry and others (1951). Assays were carried out using the conditions in Norval and others (1975). The polymerase activity after preincubation with RNase, and its sensitivity to actinomycin D was determined.

Results

Of the 14 fibroblast cell strains used in the course of this work, all were free of mycoplasma contamination except cultures A and C which were found to contain *Mycoplasma orale 1*. Testing for the isoenzyme glucose-6-phosphate dehydrogenase showed that all cell strains possessed the slow (B) type compared to HeLa cells which have a fast (A) type.

ULTRASTRUCTURE
Ultrathin sections of the cells showed highly vacuolated cells with dilated endoplasmic reticulum, shown in Fig. 1. Synovial fibroblasts from rheumatoid sources could not be distinguished from the non-rheumatoid synovial fibroblasts. The cells had an increased lipid content after storage in liquid nitrogen for a period of time in medium containing 10% dimethylsulphoxide, followed by regrowth in normal medium; otherwise no difference in structure or growth could be detected. The only strain showing any abnormality was B and, in this case, the cells contained considerable deposits of glycogen granules (Fig. 2). These were identified using the special glycogen strain of De Bruijin (1973) at the electron microscope level and could be removed by preincubation with diastase. This material appeared to be very similar to that described previously in what was presumed to be a culture arising from accidental co-cultivation of Chang and synovial cells (Mackay and others, 1974). Strain B grew very slowly compared to the other cultures, although the rate could be increased by the addition of hyaluronidase (80 units/ml) to the culture medium.

INDUCTION
Several methods were tried to induce an RNA virus, should it be present, from the integrated state. These

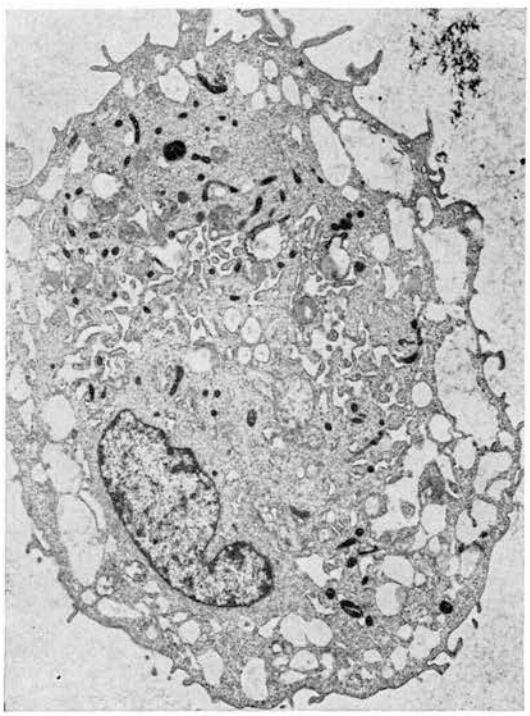

FIG. 1 *Ultrathin section of synovial fibroblast from strain I.* (×3 900)

A, D, F, I, and N. This was followed by assay of the culture supernatant for reverse transcriptase activity which would indicate release of leucovirus particles. Testosterone has been reported to induce C-type virus from the human carcinoma cell line HBT-3 after pretreatment with IUDR (Holder and others, 1974). With the synovial cells, testosterone was found to inhibit the growth of all the strains to some extent. The level used proved toxic for strain I, but the others were carried for the full 8-week period and showed no change in morphology as seen in the light microscope. At no time, except in the case of strain A, did the culture supernatant show any sign of DNA polymerase activity. With A, there was a small increase in acid-precipitable counts over the 2-hour incubation period but this is probably accounted for by cell lysis and release of cellular DNA polymerase.

Induction of strains E, F, K, and N with BUDR was also carried out, followed by assays of the culture supernatant for the presence of reverse transcriptase. Again no activity was detected.

CO-CULTIVATION

As an alternative to and in addition to induction of the synovial fibroblasts, various methods of co-cultivation were tried. Cell lines from several sources were used in an attempt to discover one which may be permissive for a defective virus. In the first place an XC test was tried with synovial strains A, D, F, I, and N. It has been shown that plaques are formed when XC cells are placed in contact with cells infected with laboratory or field isolates of murine leukaemia virus (Rowe and others, 1970). However, when the synovial cells were tried in this test, no giant cells, syncytia, or plaques were formed in the cell sheet as demonstrable by light microscopy or haematoxylin staining.

In the second place, the method of Benveniste and others (1974) by which C-type particles were induced from baboon placenta was tried. In this case the synovial cells from strains A, D, F, I, and N were induced for 24 hours with IUDR and co-cultivated with LLC-MK_2, D-17, XC, 118 MG-EH, HT-1, and human fetal skin fibroblasts. These cell lines were also cultured alone. The culture supernatants were assayed for the presence of reverse transcriptase enzyme. After incubation for 1 week, the cell lines added to the synovial fibroblast cultures were the dominant cells in the co-cultivation. The assays for reverse transcriptase of both the co-cultivated cells and the cell lines showed a slight increase in acid-precipitable counts over the 2-hour incubation period but this is probably due to the presence of DNA polymerase released by cell lysis. The rate of incorporation of the supernatant from the cell lines alone was always the same as that when co-cultivated with the synovial fibroblasts. It was therefore concluded that no C-type particles had been induced by this method, or that the cell lines chosen were not permissive for such a virus.

DNA POLYMERASE ACTIVITY

In addition to assaying the culture supernatant for reverse transcriptase activity, the synovial fibroblasts themselves were assayed. The sensitivity of the reaction to preincubation with RNase was determined. Also it is necessary, as has been pointed out previously (Norval and others, 1975), to distinguish viral reverse transcriptase within the cells from normal RNA-primed but DNA-directed DNA polymerase. Here the distinction was made on the basis of sensitivity to actinomycin D as viral reverse transcriptase is unaffected by the presence of this antibiotic, while cellular DNA polymerase is inhibited (McDonnell and others, 1970). The strains used and the results obtained are shown in Table III. It may be seen that all strains showed endogenous DNA polymerase activity. This is expressed as an increase in acid-precipitable counts per minute over the first 30 minutes of the incubation per μg protein. An enzyme concentration which gives a linear incorporation over this period was chosen. There did not appear to be a noticeable difference in the level of this enzyme in the rheumatoid cells compared to the nonrheumatoid. The activity in all strains was dependent to a large extent on the presence of RNA as shown by the sensitivity of the reaction to preincubation with RNase. However, the activity was severely inhibited by actinomycin D in all cases, which may indicate that the polymerase is RNA-primed rather than RNA-directed and hence not viral in origin.

TRANSFECTION

As a final attempt to extract a possible viral genome from the synovial cells, a transfection experiment was carried out. DNA was extracted from strains C and F and was used to 'infect' human fetal skin fibroblasts at a low pass. The cultures were split at about 10-day intervals and kept until the cells began

Table III *DNA polymerase activity of synovial strains*

Strain	*Polymerase activity (increase acid-insoluble cpm in 30 min/μg protein)*	*Activity after preincubation with RNase*	*Activity in presence of actinomycin D*
A	4·0	1·0	0
D	4·3	0·3	0·3
E	9·6	0·25	—
F	2·2	0	0
H	0·9	0	—
J	1·8	0	0·4
K	5·6	0·25	—
L	1·7	0	0·2
M	1·6	0	0
N	4·8	0	0

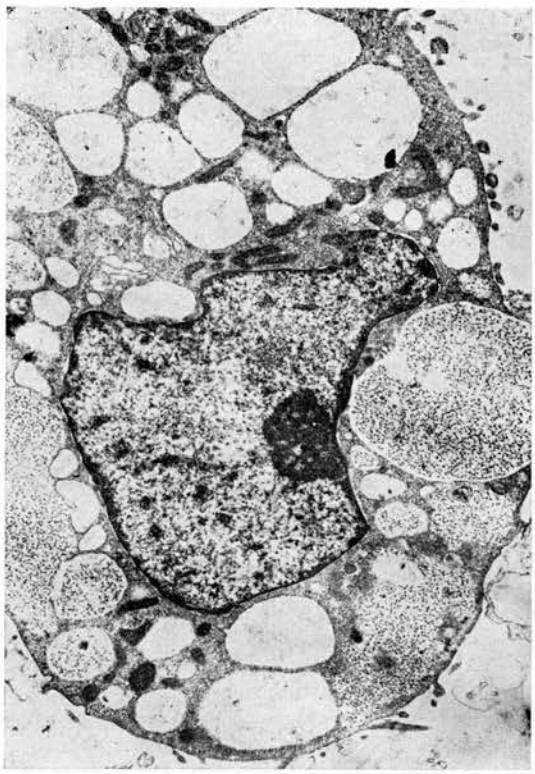

FIG. 2 *Ultrathin section of synovial fibroblast from strain B.* (×660)

methods depended on the use of various nucleic acid analogues such as IUDR, or protein inhibitors such as cycloheximide. Addition of the inducer to the medium was followed by labelling with ^{3}H-uridine, which will be incorporated into the RNA of any virus being synthesized. The medium was then concentrated by ammonium sulphate precipitation and fractionation carried out by sucrose density gradient centrifugation. Leucoviruses typically have a density of 1·16–1·18 g/cm^3 under such conditions (Robinson, 1967), while that of mycoplasmas is around 1·22 (Todaro, Aaronson, and Rands, 1971). Table II shows the cell strains which were induced and the agents used.

In all cases there was no radioactivity in the region of the gradient where leucoviruses band. Fig. 3 shows this result with strain F induced with mitomycin C. With strains A and C induced with IUDR and DMSO, there was a peak of radioactivity at a density of 1·22, as shown in Fig. 4. As these cultures are known to be contaminated with mycoplasmas which have this particular density, the incorporation of ^{3}H-uridine into acid-insoluble material seems likely to be into mycoplasma particles. As a positive control for this method of detecting RNA viruses, a culture of feline embryonic amniotic cells chronically infected with feline leukaemia virus was used. The virus particles had the appropriate density of 1·17 on the sucrose density gradient used.

In addition to the above methods, induction with testosterone was attempted using synovial cultures

Table II *Methods of induction of synovial strains*

Strain	*Induction*
B, E, F, K, N	BUDR
A, C, F, I, N	IUDR + DMSO
D, E	Cycloheximide
F, I, N	Mitomycin C
A, C, F, I, N	Testosterone

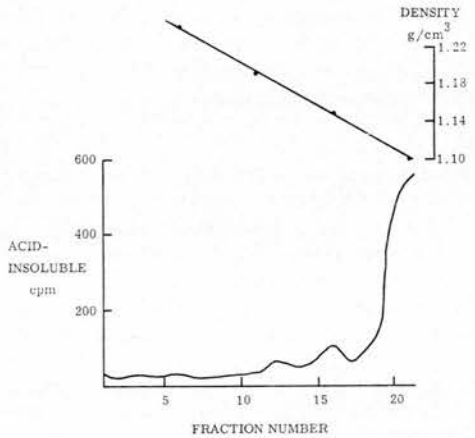

FIG. 3 *Strain F, pass 7 (induced with mitomycin C and labelled with ^{3}H-uridine): acid-insoluble cpm in fractions from sucrose gradient*

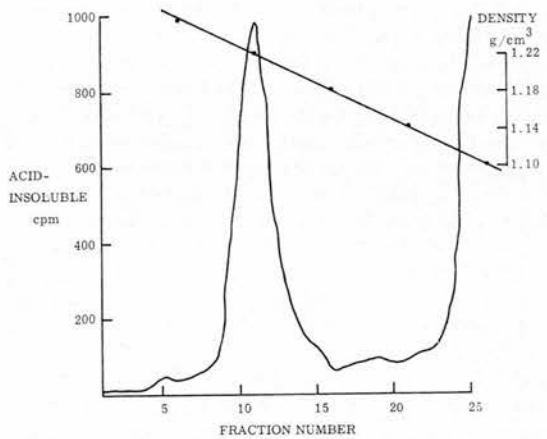

FIG. 4 *Strain A, pass 12 (induced with IUDR and DMSO and labelled with ^{3}H-uridine): acid-insoluble cpm in fractions from sucrose gradient*

An alternative approach is to look for viral-coded antigens on the cell surface and now the synovial strains are being examined for leucovirus group-specific and interspecies antigens. Apart from infection with leucoviruses, the methods used would have detected a productive infection with rubella virus. In view of the results of Patterson, Howard, and Deinhardt (1973), the synovial fibroblast strains have also been examined for rubella virus antigens by immunofluorescence and by radioimmunoassay but with negative results so far (H. Hart and B. P. Marmion, unpublished).

This work was supported by a grant from the Nuffield Foundation. We wish to acknowledge the help of Dr. M. Ogilvie and the consultant surgeons at the Princess Margaret Rose Hospital, Edinburgh, for providing the synovial membrane specimens. Synovial strains A, B, C, and D were kindly provided by Dr. D. Hamerman and most of the remainder were first cultured at the Northern General Hospital by Mr. W. A. Neill. We thank Dr. J. L. Riggs for the D-17 cell line, Dr. A. Karpas for the HT-1 cell line, Dr. O. Jarrett for the XC cell line and the feline leukaemia virus, and Dr. J. Ponten for the 118 MG-EH cell line. We are very grateful for the help of Mrs. Alexia Graham in examining the ultrastructure of the synovial fibroblast cells.

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to die after about 8 months. There was no sign of transformation and no change in cell shape or growth in the DNA-infected cultures compared to the controls.

Discussion

The synovial fibroblasts described in this work were all used at as low a subculture as possible and normally under pass 12. They began to die out around pass 18. No difference could be detected in gross morphology between strains from rheumatoid and nonrheumatoid sources or in growth rate, except that the rheumatoid cells tended to be more spaced out, perhaps due to their increased hyaluronic acid content. Also, ultrastructural studies showed no apparent differences apart from one rheumatoid strain which had significant accumulations of glycogen granules. This strain grew very slowly compared with the others and the deposits may reflect some altered cellular metabolism, although it is interesting that such deposits have been reported in a line of hamster cells (HT-1) transformed by murine sarcoma virus (Karpas and others, 1971). They have also been shown in what was presumed to be a culture arising from accidental co-cultivation of Chang and synovial cells and details of this will shortly be published (M. Norval, A. Graham, and B. P. Marmion, unpublished).

Various attempts were made to rescue a defective or integrated RNA virus which might be present within the rheumatoid synovial cells and, in particular, techniques developed for leucovirus detection were used. Induction methods included addition of IUDR, a nucleic acid analogue; cycloheximide, a protein inhibitor; mitomycin C, a DNA inhibitor; and the hormone testosterone. After treatment with these compounds for varying lengths of time, the culture supernatant was tested for the presence of RNA viruses by ^{3}H-uridine labelling, or for leucoviruses by reverse transcriptase assays. The culture supernatant from at least 5×10^{6} synovial cells was used in the labelling experiments and was concentrated by ammonium sulphate precipitation before being layered on a sucrose density gradient. 20 ml of culture supernatant was used in the reverse transcriptase assays and concentrated first by centrifugation through 20% glycerol according to the method of Lieber and others (1973). Sensitivity of the reaction was increased by the addition of polyadenylic acid-decathymidylic acid to the assay mixture. No positive results were obtained with any technique.

In addition to inducing the synovial fibroblasts, co-cultivation with a variety of cell lines was tried. It has been shown recently by Todaro and others (1974) that C-type viruses can be induced from several normal baboon tissues by co-cultivation with permissive host cell lines derived from a variety of mammalian species. They were not infectious for several baboon cell cultures. Thus it was decided to try co-cultivation of synovial fibroblasts with five cell lines from species other than man, plus human fetal skin fibroblasts. Some of the cell lines already contained part of a viral genome within the cells, such as the HT-1 cell line which consists of hamster cells transformed by murine sarcoma virus. It was thought that these might complement any viral information within the synovial cells. However all the co-cultivation methods failed to show the presence of a leucovirus or a cytopathic virus. Of course this does not mean that such a virus is definitely not present in an integrated form within the synovial cells as the methods of induction and co-cultivation may not be suitable for its expression, or the cell lines chosen may not be permissive.

Assays for DNA polymerase activity in the synovial cells failed to show the presence of viral reverse transcriptase. There was endogenous DNA polymerase activity which was shown to be RNase sensitive. However, it was strongly inhibited by actinomycin D which probably means that the enzyme is not viral in origin but is cellular RNA-primed DNA-directed polymerase. The cell lysate is a relatively crude preparation and it was found difficult to obtain sufficient cells at a low pass to try further experiments, such as the effect of various synthetic templates. However, the results obtained do corroborate the recent report of Spruance and others (1975) where cultured synovial cells from 8 patients with RA and 7 controls were examined for reverse transcriptase. On the basis of ability to utilize various synthetic templates, it was concluded that there was no viral reverse transcriptase present in any strain, and all the activity was due to DNA-directed polymerase. They reported a slightly increased level of cellular DNA polymerase in the rheumatoid cells compared to the nonrheumatoids. This was not found under the conditions used to assay this series of synovial strains, where no exogenous template was added. Again, finding no reverse transcriptase enzyme does not exclude the presence of a leucovirus genome from the synovial cells as this enzyme may not always be expressed or may not be detectable under the assay conditions used.

In a final attempt to rescue a leucovirus genome, the nuclear DNA from a rheumatoid and a nonrheumatoid strain was extracted and used in a transfection experiment with human fetal skin fibroblasts. Recently it has been shown that cellular transformation can be induced by viral-coded DNA, in particular DNA extracted from human rhabdomyosarcoma cells caused transformation of fetal skin fibroblasts (Karpas and Tuckerman, 1974). However, no transformation or change in cellular morphology took place over an 8-month period.

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Cytology of rheumatoid synovial cells in culture

IV. Further investigations of cell lines cocultivated with rheumatoid synovial cells

MARY NORVAL, ALEXIA GRAHAM, AND B. P. MARMION

From the Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH9 9AG

Norval, M., Graham, A., and Marmion, B. P. (1976). *Annals of the Rheumatic Diseases*, **35**, 297–305. **Cytology of rheumatoid synovial cells in culture. IV. Further investigations of cell lines cocultivated with rheumatoid synovial cells.** A previous report described a cell isolate presumed to have arisen by accidental cocultivation (contamination) of the Chang 'liver' cell line and rheumatoid synovial cells. This cell isolate had the same glucose-6-phosphate dehydrogenase isoenzyme as the Chang cell and also some shared antigens. It clearly differed in its karyotype, its ability to grow in semisolid agar, and in the possession of bleb-like projections of the cytoplasmic membrane filled with collections of beaded or granular material. In addition, it had a novel antigen(s) not present in the Chang cell.

As these properties might have been acquired from the synovial cells and because the bleb structures resembled those seen in some cell lines transformed by leucovirus the cell isolate has been further studied. Cytochemical methods at the light and electron microscope level showed that the granular material was polysaccharide in nature, probably glycogen. No evidence was found of the presence of a virus or a viral genome using a variety of techniques including attempted induction followed by ^{3}H-uridine labelling of the cultures, and assay of the supernatant fluid from the culture for viral RNA-dependent DNA polymerase. In addition, cell extracts were not found to contain viral RNA-dependent DNA polymerase or RNA-dependent RNA polymerase. No rubella virus or leucovirus interspecies antigens were detected on the cell membranes.

Mackay and others (1974) and Mackay, Marmion, and McCormick (1975) reported the isolation of an epithelial cell line, 710025, which had apparently arisen from a culture of rheumatoid synovial cells and gave reasons for concluding that it was probably the result of a laboratory contamination of the synovial culture with Chang 'liver' cells. However, the cell line from this unintentional cocultivation had features that differed from the presumed parent, the Chang cells. First, there was a difference in karyotype, in particular the presence of an additional satellite chromosome in the 710025 cells. Second, the 710025 cells were able to form small colonies in semisolid agar whereas the Chang cells did not. Third, sera from a proportion of patients with rheumatoid or nonrheumatoid arthritis reacted by membrane immunofluorescence with Chang and 710025 or equivalent cells but absorption analysis indicated that the latter had additional, unique, antigens to those shared with Chang cells; this point has been substantiated by antigenic analysis with antisera prepared in rabbits rendered tolerant to Chang cells (J. N. McCormick, personal communication). Finally, ultrastructional examination of the cells showed bleb-like projections of the cytoplasmic membrane—not seen in the Chang cells—and in some of the cells there were extensive collections of beaded or pseudofilamentous structures, either deep in the cytoplasm or in the blebs, or in both places.

Two additional cell lines, 720151 and 720301, with essentially similar characteristics were isolated

Accepted for publication January 5, 1976.
Correspondence to Dr. M. Norval.

shortly afterwards, presumably again from contamination, this time with 710025 cells. Since this period, with added precautions against contamination, some 150 synovial membranes or exudates from rheumatoid or nonrheumatoid patients have been examined in culture without isolation of any 'transformed' cell lines. Efforts to reproduce the phenomenon by deliberate cocultivation of freshly taken synovial exudates and Chang cells were unsuccessful (Mackay and others, 1975); in many instances the Chang cells were eliminated by macrophages in the cultures and the survivors did not show the blebs or fibrillary material.

The epithelial cell isolates, typified by 720301 cells, required further investigation for a number of reasons. The cytoplasmic fibrillary material superficially resembled paramyxovirus nucleocapsids, or, alternatively, glycogen, and it was important to establish its nature. The bizarre morphology of 720301, and the related cell lines, resembled similar cytology in human pleural mesotheliomas (Davis, 1974), and that in a variety of tumour or transformed cells resulting from infection with avian or murine leucoviruses. Thus, for example, surface blebs and accumulations of glycogen, together with distinctive, additional, marker chromosomes, have been observed in a line of hamster cells (HT–1) transformed by murine sarcoma virus (Karpas and others, 1971); virus particles and murine leukaemia group antigen were not detected in those cells.

This paper reports the results of investigations designed to define the nature of the beaded or pseudofilamentous structures in the cocultivated cells, and attempts to detect the genome or gene products of rubella, paramyxovirus or leucovirus.

Materials and methods

CULTURE METHODS

Cell lines were grown in Eagle's complete medium (1959 modification) containing 5% calf serum normally in Roux bottles or on cover-slips in tubes at a temperature of 37°. They were passed by trypsinization.

CYTOCHEMISTRY

For light microscopy, 720301 and Chang cells were removed from Roux flasks using glass beads, pelleted in Difco agar (cooled to 48°) and fixed in Bouin's fixative (Gurr, 1957). Paraffin sections were prepared, some being treated with 1% diastase (BDH) for 1 hour at 37°. The sections were stained with Schiff (Gurr, 1957) and Best's Carmine (Gurr, 1957). Cells grown on cover-slips were also stained similarly.

For electron microscopy, 720301 and Chang cells were removed from Roux flasks with glass beads. They were stained specifically for polysaccharides according to the methods of De Bruijin (1973) and Schaff, Barry, and Grimley (1973). The blocks were sectioned on an LKB Ultratome 11 and the sections viewed on a Hitachi HU11A electron microscope. Some preparations were stained by the ruthenium red method (Vorbrodt and Kroprowski, 1969) to detect differences in surface carbohydrate 'coat'.

Chang and 720301 cells were also examined for content of carbohydrate by growth for 24 hours in Eagle's medium containing either no glucose or increasing concentrations of 1 mmol/l, 5 mmol/l, and 10 mmol/l glucose. They were then harvested, solubilized with 30% potassium hydroxide, and the polysaccharide concentration of the cell-free extracts determined colorimetrically by the anthrone-sulphuric acid method of Shetlar (1952).

ATTEMPTS TO INDUCE CELLS TO PRODUCE VIRUS

(*a*) In various regimens 720301 cells were cultured in medium containing BUDR 20 μg/ml for 3 days; or IUDR 20 μg/ml for 3 days (Lowry and others, 1971); or IUDR 20 μg/ml for 3 days followed by DMSO (2%) for 4 days (Stewart and others, 1972); or in medium without arginine for 4 days (Kaplan, Wilbert, and Black, 1972). Cells were removed with glass beads, fixed in Karnosky's fixative, embedded in Araldite, and the sections examined in the electron microscope. In addition, washed cells were lysed by freezing and thawing and the lysates used to inoculate BHK-12, RK_{13} and Vero cells growing on cover-slips. Cover-slips were stained with Giemsa after 4 and 11 days' incubation.

(*b*) 720301 and Chang cells of various passage levels, in the former ranging from 7 to 28 passages, were incubated in the presence of BUDR 20 μg/ml for 3 days; or IUDR 20 μg/ml for 4 days; or IUDR 20 μg/ml for 3 days followed by DMSO 2% for 3 days; or puromycin 10 μg/ml for 1 day (Aaronson and Dunn, 1974); or testosterone acetate 5 μg/ml for 3 days (Holder, Robey, and Vande Woude, 1974); or mitomycin C 1 μg/ml for 18 hours in the dark (Weiss and others, 1971). The attempted induction was followed by ^{3}H-uridine labelling as outlined by Norval and Marmion (1976). For one experiment, after treatment with testosterone, 720301 cells were incubated in medium containing 2·5 μCi/ml (methyl–^{3}H) thymidine and 0·4 μCi (2–^{14}C) uridine (Amersham).

DNA POLYMERASE ACTIVITY

(*a*) *Preparation of cells and polymerase assay*

720301 and Chang cells were washed in medium and prepared as outlined by Norval, Ogilvie, and Marmion (1975).

(*b*) *Preparation of culture supernatants and polymerase assay*

20 ml of culture supernatant from confluent 720301 and Chang cultures, either before or after attempted induction with BUDR and testosterone acetate were prepared according to the method of Lieber and others (1973), and the sedimented pellet resuspended in 0·1 ml polymerase buffer as used in (*a*). To this was added double quantities of the assay constituents and incubation carried out at 37° for 2 hours. Samples of 50 μl were spotted on filter paper discs at 30-minute intervals and acid-precipitation and counting performed as above.

MEMBRANE ANTIGENS

720301 and Chang cells were examined for interspecies antigens associated with mammalian leucoviruses by membrane immunofluorescence following the method of Yoshiki and others (1974). The antisera used were rabbit antifeline leukaemia virus, HCl-guanidine disrupted (kindly provided by Dr. O. Jarrett), goat antifeline leukaemia virus p27 and p12, and goat anti-Mason Pfizer monkey virus p27 and p10–12 (kindly provided by the National Cancer Institute). The cells were also examined for rubella antigens using the method described by Hart and Marmion (1976).

Results

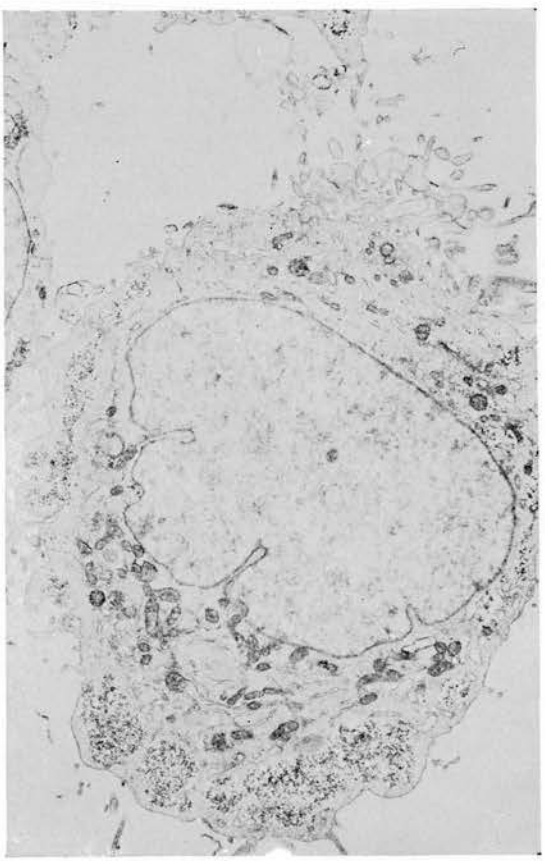
PSEUDOFILAMENTOUS CELLULAR INCLUSIONS

Cytochemical techniques were used in attempts to identify the material within the bleb-like structures and to distinguish glycogen granules and nucleocapsids of incomplete viral structures containing nucleic acid. Most of the experiments with Chang and 720301 cells were done with cultures infected with *Mycoplasma orale* 1 (Mackay and others, 1974). However, a subline of 720301 cells which had been freed of mycoplasmas by 'pulse' treatment with sodium aurothiomalate and *M. orale* 1 antiserum and complement was used in ultrastructural studies.

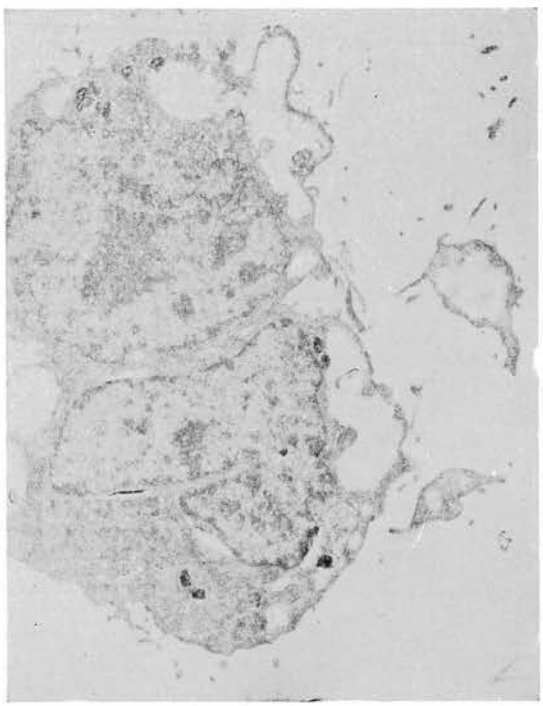
Paraffin sections of pellets of 720301 cells, stained for polysaccharide, showed Schiff-positive material occurring in large particulate areas in the cytoplasm. There were also masses of this material outside the cells. On the other hand, Chang cells only occasionally showed traces of Schiff-positive material and small amounts extracellularly. Best's carmine staining of paraffin sections gave less striking results but the majority of 720301 cells seemed to have some glycogen, whereas very few Chang cells contained glycogen.

On the other hand cover-slip cultures of 270301 cells in monolayer showed much less Schiff-positive material in the form of discrete inclusions. Thus the difference in polysaccharide content with Chang and 720301 cells was less marked in cover-slips than in the paraffin sections, even so there were more Schiff-positive 720301 cells than Chang cells.

The results of the tests at the light microscope level were confirmed by ultrastructural examination.



a



b

FIG. 1 *Ultrathin section of 720301 cell stained with* $K_3Fe(CN)_6$ *to show (a) glycogen granules in the cytoplasm; (b) absence of glycogen granules after pretreatment with diastase.* ×440

The results with the technique of De Bruijin (1973) with $K_3Fe(CN)_6$ are illustrated in Fig. 1*a* and 1*b*. Approximately 70% of the 720301 cells showed large stained areas in the cytoplasm which were removed after incubation with diastase, but not if the enzyme had been inactivated first by boiling. The same result was obtained after the 720301 cells had been freed of Mycoplasma contamination. The stained areas in the Chang cells were much smaller and were found in only about 40% of the cells. Finally, chemical analysis of cell lysate showed consistently higher levels of glycogen in 720301 cells compared with Chang cells, although in each instance higher levels of storage were attained if the concentrations of glucose in the culture medium was increased (Table). Ruthenium red staining did not show a difference in cell 'coat' between Chang and 720301 cells.

Table *Analysis of glycogen content (estimated as glucose in hydrolysed cell lysate) of 720301 and Chang cells grown in Eagle's medium with varying content of glucose*

Cell	*Glucose content of medium (mmol/l)*	*μg glycogen/ mg protein*
720301 pass 19	—	68
	1	72
	5	107
	10	150
Chang pass 284	—	20
	1	19
	5	45
	10	50

INVESTIGATION FOR INFECTIVE VIRUS OR VIRAL GENE PRODUCTS

(*a*) *Infection with cytoplasmic DNA viruses and RNA viruses, other than leucoviruses*

As previously reported (Mackay and others, 1974) paramyxovirus (measles, mumps, and parainfluenza) antigens were not detected in cell lysates of 720301 by complement fixation reactions with known antisera. Rubella antigen was not detected by membrane or other immunofluorescence with rabbit antisera that reacted satisfactorily with LLC-MK_2 cells chronologically infected with rubella virus (Hart and Marmion, 1976). In addition immunization of rabbits with cell suspensions did not stimulate viral antibodies.

Lysates of 720301 and Chang cells were also assayed for RNA-dependent RNA polymerase using the method of Scholtissk and Rott (1969). Results were negative but activity was detected in the corresponding lysates from LLC-MK_2 cells chronically infected with rubella and PK_{15} and MDBK cells chronically infected with Newcastle Disease virus (kindly provided by Mr. E. Gowans).

(*b*) *Infection with leucoviruses*

In view of the striking blebs and glycogen accumulation and the observations that cells with leucovirus genomes sometimes exhibit such morphological changes, extensive attempts have been made to induce the 720301 cells to form complete virions or gene products such as RNA-dependent DNA polymerase or to show interspecies antigens characteristic of the mammalian leucoviruses.

INDUCTION EXPERIMENTS

Many methods have been described in recent years whereby viruses have been induced from an integrated state to form complete virions. In particular, activation of RNA-tumour viruses in various situations including cell lines from human sources has been reported (Stewart and others, 1972; Holder and others, 1974; Bykovsky and others, 1973). RNA tumour viruses thus produced have a density of 1·16–1·18 g/cm^3 (Robinson, 1967) on sucrose gradient equilibrium centrifugation, while Mycoplasmas have a density around 1·22 g/cm^3 (Todaro, Aaronson, and Rands, 1971).

Attempts were made to induce the 720301 cells by several methods, including BUDR, IUDR, IUDR followed by DMSO, puromycin, and testosterone acetate. In the first instance ultrathin sections were examined in the electron microscope. The presence of the inducers had a general harmful effect on various cell structures except where DMSO was used in addition to IUDR. Puromycin caused an inhibitory effect with cessation of growth and rounding of cells. Testosterone had an even more toxic effect with much of the cell sheet detaching from the glass over the 3-day incubation period. No virus particles were seen and the bleb-like areas did not alter in shape and frequency. The Chang cells were noticeably less affected with all treatments including testosterone.

In addition, washed cells after induction were lysed by freezing and thawing, and the effect of the lysates on BHK-21, RK_{13} and Vero cells growing on cover-slips found. There was no indication of virus infection as demonstrable by Giemsa staining.

As an alternative method for the detection of RNA virus virions, the cultures were labelled with tritiated uridine and the labelled constituents of the concentrated culture supernate separated by sucrose gradient equilibrium centrifugation. As a positive control for these experiments feline leukaemia virus (FeLV-B) grown in feline embryo cells was labelled with 10 μCi/ml of ^{3}H-uridine. This had a density of 1·16–1·17 g/cm^3 after sucrose gradient density centrifugation as outlined in Methods.

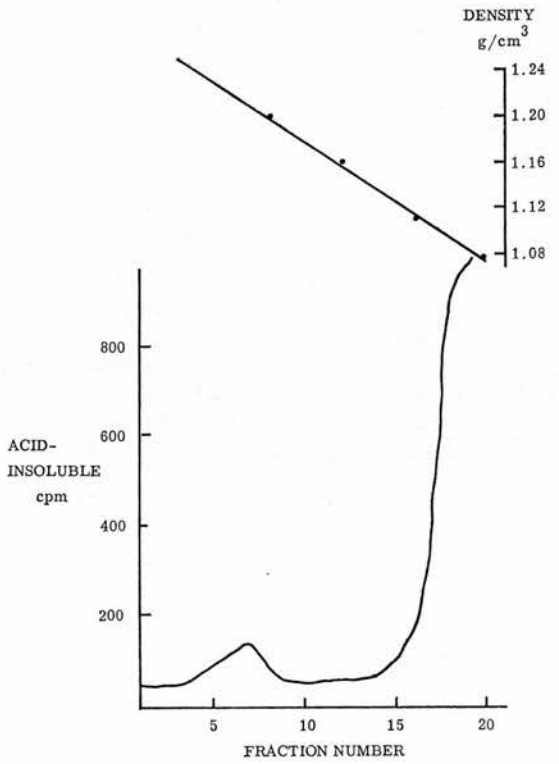


FIG. 2 *Acid-insoluble cpm in sucrose density gradient fractions from 720301 culture supernatant, labelled with ^{3}H-uridine as in Methods*

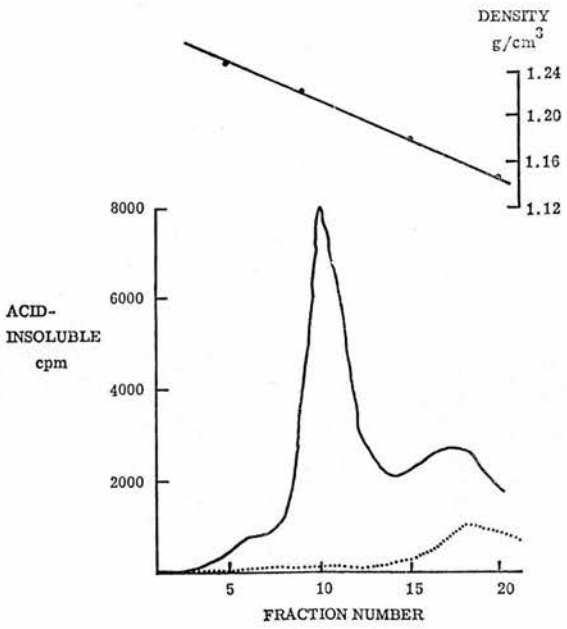


FIG. 3 *Acid-insoluble cpm in sucrose density gradient fractions from 720301 (——) and Chang (· · · ·) culture supernatant, induced with testosterone before labelling with ^{3}H-uridine*

A double-labelling experiment after testosterone induction using ^{3}H-thymidine and ^{14}C-uridine showed that the peak at density around 1·21 contained both DNA and RNA (Fig. 4). *Mycoplasma orale* 1 was cultured on cell-free medium from an equivalent fraction containing this material. Thus it seems that the 720301 cells are not producing an RNA virus even after induction, and that the incorporation of labelled uridine and thymidine into cellular material is into Mycoplasmas.

When 720301 cells were treated similarly, there was a small peak of radioactivity around density 1·22, but no evidence of any incorporation of ^{3}H-uridine at density 1·16–1·18 (Fig. 2). This peak was enlarged by the presence of any of the inducers listed in the Methods but particularly by treatment with testosterone acetate (Fig. 3). On the other hand, the Chang cells showed no incorporation at this density even after induction with testosterone (Fig. 3).

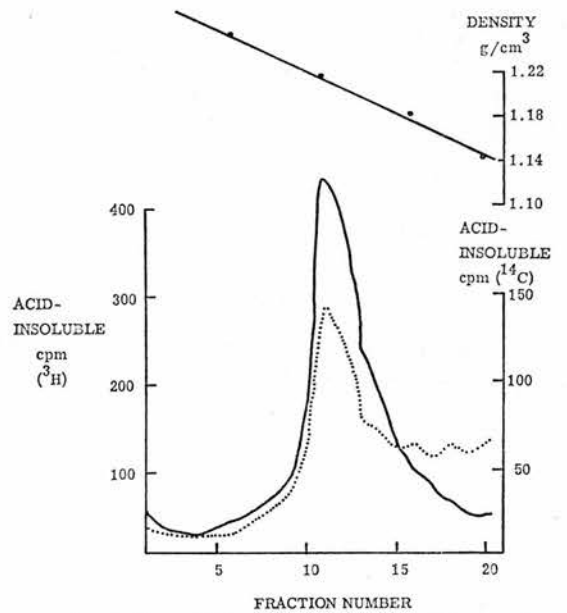


FIG. 4 *Acid-insoluble cpm in sucrose density gradient fractions from 720301 culture supernatant, induced with testosterone before double labelling with ^{3}H-thymidine (——) and ^{14}C-uridine (· · · ·)*

ASSAY FOR RNA-DEPENDENT DNA POLYMERASE AS AN INDICATOR OF LEUCOVIRUSES

Enzyme extracts were prepared from lysed cells and tested for the presence of endogenous RNase-sensitive DNA polymerase. Fig. 5 shows a typical result using 10 μl of Chang and 720301 preparations. The enzyme activities may be expressed as an increase in acid-precipitable counts/min over the first 30 minutes of the incubation per μg protein. For 720301 cells the result is 6·1; for Chang cells 7·6.

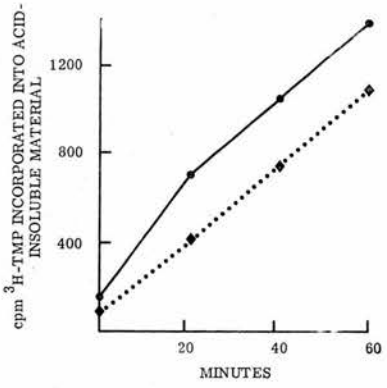


FIG. 5 *Endogenous DNA polymerase activity of 720301 and Chang cells. Assay conditions as in Methods, using 10 μl crude enzyme preparation*

The product of the reaction was completely sensitive to DNase in both cases as shown by the reduction in acid-insoluble counts to background level after treatment with DNase at the end of the incubation period (Fig. 6; 720301 cells). The dependence of the polymerase activity on the presence of endogenous RNA is shown by pre-incubation of the enzyme preparation with RNase (Fig. 6).

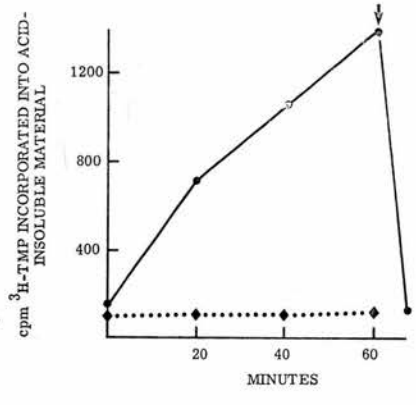


FIG. 6 *Effect of preincubation with RNase (◆) and post-incubation with DNase on endogenous DNA polymerase activity of 720301 cells. Assay conditions as in Methods using 10 μl crude enzyme preparation and 10 μl after preincubation with RNase. DNase was added (arrow)*

Various methods have been described, such as by Norval and others (1975) to distinguish the RNA-dependent DNA polymerase characteristic of leucoviruses from other cellular polymerases, in particular the DNA polymerase primed by RNA but directed by DNA, found in normal stimulated human lymphocytes (Bobrow and others, 1972). Thus, the sensitivity of the enzyme from the Chang and 720301 cells to actinomycin D was ascertained. The result (Fig. 7) for 720301, and the Chang enzyme was the same. The antibiotic inhibited the reaction by over 90% even for the first sample taken 5 minutes after the start of the incubation period. Secondly, the ability of the polymerase to utilize the synthetic templates poly-d(A-T) and poly-A·d(T)$_{10}$ was determined. It was found that poly-d(A-T) stimulated the activity over 15-fold while poly-A·d(T)$_{10}$ had no effect.

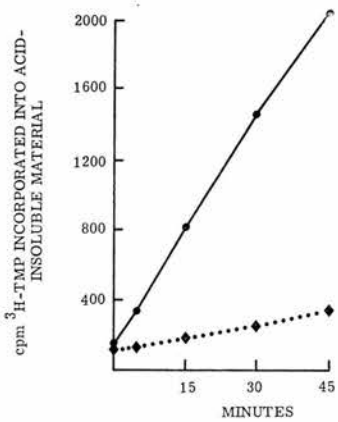


FIG. 7 *Effect of actinomycin D on endogenous DNA polymerase activity of 720301 cells. Actinomycin D (50 μg/ml) dissolved in polymerase buffer was added to the assay mixture (◆) or polymerase buffer alone (●).*

Attempts were also made to analyse the product of the endogenous reaction in caesium sulphate and glycerol gradients. All the radioactivity was at the appropriate density for DNA, that is around 1·41 on Cs_2SO_4 equilibrium density centrifugation and near the top of the glycerol gradient. There was never any evidence of the formation of a labelled RNA-DNA hybrid which should band to a density of around 1·66 g/cm^3 on Cs_2SO_4 (Sarngadharan and others, 1972), and have sedimentation coefficient of 70S on a glycerol gradient (Gulati, Axel, and Spiegelman, 1972).

The results therefore do not indicate the presence of a viral RNA-dependent DNA polymerase in the cell lysates. As a positive control in these experiments, use was made of the cell line 118MG-EH containing the Rous sarcoma virus genome with expression of reverse transcriptase activity (Norval and others 1975).

Also assay of the culture supernatants of Chang and 720301 cells did not indicate the presence of a viral reverse transcriptase. There was a very slight increase in acid-precipitable counts over the 2-hour incubation period in the case of the 720301 cells. This was not increased after induction with BUDR but did increase slightly after treatment with testosterone. The raised activity is probably due to the release of DNA polymerase by cell lysis or death and is certainly not sufficiently high to indicate budding of RNA tumour virus into the culture medium as shown by Lieber and others (1973), even in the presence of poly-A·d $(T)_{10}$ in the assay mixture.

LEUCOVIRUS ANTIGENS

Although mature leucovirus particles may never be formed by an infected cell line, there may still be expression of some viral antigens in the cell, such as the interspecies antigen associated with the major core protein p27 of the virus. These interspecies antigens were thus looked for in 720301 and Chang cells by immunofluorescence (Yoshiki and others, 1974) using antiserum prepared against HCl-guanidine disrupted feline leukaemia virus. In addition, antisera prepared against p27 and p12 proteins of feline leukaemia virus and Mason-Pfizer monkey virus were tried. There was no staining of either cell line with any of the antisera.

TRANSFECTION AND XC TEST

Two final experiments were tried in an attempt to show leucovirus information within the cells. It has been reported that DNA extracted from a human rhabdomyosarcoma cell line caused transformation of human fetal fibroblasts (Karpas and Tuckerman, 1974). Nuclear DNA was therefore extracted from the 720301 and Chang cells and used to 'infect' fetal skin fibroblasts which were split at intervals until they began to die out after about 8 months. There was no change in cell shape, growth, or other evidence of transformation.

Lastly an XC test was carried out. It has been shown that, when cells containing murine leukaemia virus genomes were overlaid with XC cells, syncytia and plaques developed in the cell sheet (Rowe, Pugh, and Hartley, 1970). No effect of this nature was observed in the case of the 720301 or Chang cell lines.

Discussion

The cell lines described in this paper were assumed to have arisen by accidental cocultivation of the Chang cell line with synovial fluid cells. As viruses have been proposed as infective agents in rheumatoid arthritis it was therefore of interest to discover if the unusual characteristics of these cells could be attributed to a viral infection of some kind—either productive and noncytopathic, or defective in some way, or integrated.

One of the most striking features of these cells was the bleb-like projections of the cytoplasmic membrane with associated accumulations of granular material in the cytoplasm. Originally it was though that this material might be nucleocapsid in origin at occurs during chronic infection of cells with News castle disease virus (Mackay and others, 1974), but subsequently the results of various staining methods at the light and electron microscope level showed that the material was polysaccharide, probably glycogen. This was interesting as glycogen can accumulate in cells infected with either Mycoplasmas or leucoviruses. To resolve this difficulty, the cell line 720301 was cleared of Mycoplasma contamination by pulse treatment with sodium aurothiomalate and *M. orale* 1 antiserum plus complement. No Mycoplasmas were obtained on subsequent subculture and immunofluorescence using goat anti-*M. orale* serum was negative. However after this treatment the cells were shown to contain glycogen accumulations by electron microscopy.

No virus particles have been seen budding from the cell membrane or associated with the cell, and there was no evidence of a productive viral infection of any kind. Paramyxovirus antigens were not detected in cell lysates nor rubella antigens at the cell surface. It is known that leucoviruses can exist in an integrated state within the host cell's DNA, and may express some of their viral genes by the synthesis of RNA-dependent DNA polymerase or viral-coded antigens. Some may be induced by a variety of agents to complete the replicative cycle and to bud from the cell membrane into the culture supernatant. Several different inducers were therefore tried and the effect of these found by electron microscopy, by treatment of other cell lines with cell lysates, and by labelling with ^{3}H-uridine and thymidine followed by sucrose gradient centrifugation. With the latter method, the results were complicated by the presence of Mycoplasmas which seemed to be released from the cell membrane after induction. However, they could be separated from leucoviruses as Mycoplasmas have a higher density and could be labelled with both radioactive uridine and thymidine. No peak of radioactivity with density appropriate for a leucovirus was found. The number

of Mycoplasmas in the culture supernatant appeared to be increased by treatment with inducers, or perhaps more are liberated from the cell by membrane effects, particularly with testosterone. The Chang cell line seemed to be much more stable than the 720301 cells in all respects, and despite containing *Mycoplasma orale* 1, did not release it in detectable quantities into the medium, even after induction. This may also be a reflection of relative population numbers.

The culture supernatant was examined for the presence of RNA-dependent DNA polymerase but none was found, even after induction with BUDR or testosterone acetate. The endogenous DNA polymerase within the cells was shown to have the characteristics of a DNA-directed and not RNA-directed enzyme. In addition no leucovirus interspecies antigen was detected on the cell membrane. Both a transfection experiment and an XC test were not successful.

With regard to RNA viruses other than leucoviruses, no RNA-dependent RNA polymerase was found in cell lysates, and tests for rubella specific antigens on the cell surface were negative.

Thus by all the techniques mentioned above, no evidence was obtained that there was a viral genome, in particular a leucovirus, present in the 'cocultivated' cell lines. The accumulation of glycogen granules remains to be explained, although it is still possible that such a genome is there but is not expressed as mature virus particles, or even as new viral-coded membrane antigens or polymerase enzymes necessary for viral replication.

We thank Dr. Barbara Marczynska for helpful discussion regarding the morphology of leucovirus-transformed cell lines. This work was supported by a grant from the Nuffield Foundation.

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Annals of the Rheumatic Diseases, 1979, **38**, 456–462

Search for viral nucleic sequences in rheumatoid cells

MARY NORVAL[1] AND CAROL SMITH[2]

From the [1]Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, and the [2]Department of Medicine, Montefiore Hospital and Medical Center, Bronx, New York

SUMMARY DNA and RNA were extracted from synovial membranes, synovial fibroblast cells, peripheral blood lymphocytes, and synovial fibroblast cells strains derived from patients with rheumatoid arthritis and other joint conditions. They were hybridised after immobilisation on nitrocellulose filters with iodinated viral nucleic acids extracted from measles, rubella virus, SV–40, and a retrovirus, RD–114. In addition, in-situ hybridisation was carried out on sections of synovial membranes by means of iodinated measles and rubella virus RNA. In no case did any hybridisation occur. Positive control systems included synovial fibroblast strains transformed with SV–40, LLC–MK_2 cells chronically infected with rubella virus and RD cells infected with RD–114. It was concluded that the synovial cells did not contain viral genomes of measles, rubella virus, SV–40, or RD–114, or at least at a level equivalent to the positive control cells.

In recent years many attempts have been made to isolate a virus from tissues of rheumatoid patients and there have been no positive results; for reviews see Marmion and Mackay (1977) and Hamerman (1975). However, the immunological abnormalities apparent in this disease could be associated with a virus which is not fully expressed and which therefore would not be detectable by virus culture methods. Such a virus might be present within the cells of the synovial membrane or synovial fluid and might alter the surface properties of these cells. Some work has been done lately to examine this possibility and again the results are predominantly negative.

Hart and Marmion (1977) studied fibroblast strains which grew in culture from synovial membranes or synovial fluids for retroviral and rubella viral antigens by immunofluorescence and radioimmunoassay and none were found, though there is evidence from Patterson *et al.* (1973) that by means of a cytotoxic assay the rheumatoid cells may be shown to contain rubella virus membrane antigens. Similarly the lymphocytes from rheumatoid synovial fluids and cells in sections of synovial membranes showed no sign of possessing retroviral or rubella viral antigens by immunofluorescence, immunoperoxidase, and radioimmunoassay (Hart and Marmion, 1977).

Attempts to rescue viruses from synovial fibroblast strains by such procedures as chemical induction and cocultivation were not successful (Norval and Marmion, 1976). Recently synovial lymphocytes were fused and cocultivated with cell lines permissive for primate retrovirus but with no virus being induced (Norval *et al.*, 1978, to be published).

Thus in the aetiology of rheumatoid arthritis there is little to implicate viruses, whether being produced from infected cells or even in a non-productive form so far. However, consideration should be given to those viruses which may be present within cells and which may alter cell function or induce new proteins at the cell surface. Such hypotheses, among others, have been reviewed recently by Marmion and Mackay (1977). For example, adenovirus may be considered as an oncogenic virus in a transforming situation where a part of the viral genome may integrate into the host cell chromosome. This cannot be recovered as infectious virus and will be detectable only by nucleic acid hybridisation or perhaps by synthesis of a few early proteins like T-antigen, although in some cell lines even these may not be expressed; for review, see Flint (1977).

We decided therefore to examine nucleic acids from rheumatoid and nonrheumatoid sources for homology with various viral nucleic acids. DNA and RNA were extracted from synovial membranes, synovial fluid cells, peripheral blood lymphocytes, and synovial fibroblasts grown in culture. These were hybridised with iodinated viral nucleic acids from measles, rubella, SV–40, and RD–114, an endogenous feline retrovirus, which is antigenically related to viruses from certain nonhuman primates, by a

Accepted for publication 1 November 1978.
Correspondence to Dr M. Norval.

simple method of fixing the cellular nucleic acid on nitrocellulose filters.

Materials and methods

PREPARATION OF CELLULAR NUCLEIC ACIDS

Specimens from synovectomies were received in the laboratory less than 2 hours after operations. The synovial membranes were dissected out and cut into small pieces (2–5 mm). They were disaggregated in serum-free medium either with trypsin or with a mixture of collagenase (1 mg/ml) and hyaluronidase (80 units/ml) at 37°C for 3 hours with stirring. The dispersed cells were washed in serum-free medium (Earles-based Eagles), then in extraction buffer (0·15M NaCl, 0·1M EDTA in 0·1M Tris, pH 8·0).

Synovial fluids, removed by syringe from various effusions, were treated with hyaluronidase (80 units/ml) for 30 mins at 37°. The cells were deposited by centrifugation and washed in serum-free medium, then in extraction buffer.

Peripheral blood lymphocytes were prepared from 100 ml heparinised blood by separation on Triosil-Ficoll gradients. They were washed in serum-free medium, then in extraction buffer.

Synovial fibroblast strains, grown in culture (Norval and Marmion, 1976) were harvested by trypsinisation and washed in extraction buffer. These were normally around pass 5.

In all cases a minimum of 2×10^7 cells were obtained.

Nucleic acid was extracted by the method outlined in Simpson and Iinuma (1975), a 10% solution of sodium dodecyl sulphate (SDS) in extraction buffer being added to make the final concentration 1%. The precipitated nucleic acid after chloroform/isoamyl alcohol extraction was treated with RNase (50 μg/ml, pretreated at 80° for 10 min, Sigma) for DNA preparations or with DNase (20 μg/ml, Sigma) for RNA preparations. The final precipitation of nucleic acid was with ethanol, and it was dissolved in SSC buffer (0·15M NaCl, 0·015 M Na citrate). The ratio of absorbance at 260 to 280 nm was at least 1·85, and from 10^8 cells the yield varied from around 100 μg in the case of synovial membrane cells to around 500 μg from cultured synovial cells. The nucleic acid was stored at −70°C until use.

In addition RNA and DNA were extracted from BHK–21 cells 3 days after infection with rubella virus (Thomas strain), and from LLC–MK_2 cells chronically infected with rubella virus (Hart and Marmion, 1977). DNA was extracted from RD virus and RD cells chronically infected with RD–114 (McAllister *et al.*, 1972). Also DNA was extracted from synovial fibroblast strains, 2 rheumatoid and 1 non-rheumatoid, transformed by SV–40. This transformation was achieved by pretreating the cells with hyaluronidase (1 mg/ml) for 1 h at 37°C, then infecting with virus at a multiplicity of 100:1 or 300:1. Transformation became apparent by morphological changes after 4 to 16 weeks, and the presence of T antigen was confirmed by immunofluorescence.

PREPARATION OF VIRUSES AND VIRAL NUCLEIC ACID

Purified SV–40 virus was a kind gift from Dr Ching-Juh Lai (National Cancer Institute).

Measles virus (Schwartz strain) was grown in Vero cells in roller bottles. The medium was Earles-based Eagles containing 2% calf serum and 100 IU/ml penicillin and 100 μg/ml streptomycin. The culture supernatant (3 litres) was harvested 3 days after infection and clarified The virus was concentrated by centrifugation at 30 000 r.p.m. for 1 hour in a SW 40 rotor, and resuspended in 0·01 M Tris-HCl, 1 mM EDTA, pH 7·1 buffer (Grayzel, 1973). It was layered on to sucrose density gradients (20% and 70%) and centrifuged at 25 000 r.p.m. for 18 hours in a SW 40 rotor. Drops were collected from the base of the tube, and fractions with density 1·19 to 1·23 g/cm^3 were pooled and pelleted at 40 000 r.p.m. for 1 hour in an SW 50·1 rotor. The pellet was suspended in 3 ml SSC and stored at −70°C.

Rubella virus (Thomas strain) was also grown in Vero cells in roller bottles. The culture supernatant (2 litres) was harvested after 5 days and 8 days and clarified. It was purified as for measles except that the 1·18 to 1·21 g/cm^3 band was taken from the sucrose density gradients.

RD cells chronically infected with RD–114 virus were grown in roller bottles in Eagles medium containing 5% fetal calf serum. Culture supernatants were harvested every second day (2 litres) and clarified. The virus was purified as for measles except that the 1·15 to 1·17 g/cm^3 band was taken from the sucrose density gradients.

The nucleic acid was then extracted from each virus preparation. The volume was made up to 10 ml in TNES buffer (0·1M Tris pH 9·0, 0·1M NaCl, 1mM EDTA, and 0·1% SDS) and extracted 3 times with TNE (0·1M Tris pH 9·0, 0·1M NaCl, 1mM EDTA)–saturated phenol containing 10% chloroform. The nucleic acid was precipitated with ethanol and the final precipitate dissolved in 0·3 ml sterile SSC and stored at −70°C.

IODINATION OF VIRAL NUCLEIC ACIDS

200 μg measles RNA, 50 μg SV–40 DNA, 200 μg rubella RNA, and 280 μg RD–114 RNA were

iodinated by modification of the method of Commerford (1971). DNA, when used, was heated at 80°C for 20 min just before iodination. A mixture was made of 0·3 ml of 0·5 M Na acetate buffer, pH 5·0; 0·3 ml SSC containing the nucleic acid to be iodinated; 0·3 ml thallic chloride, 2·5 mM in 0·1 M acetate buffer, pH 5·0; and 0·3 ml potassium iodide 0·125 mM, which had previously been added to 600 μCi ^{125}I. It was incubated at 60° for 1 hour, then cooled to room temperature. The labelled nucleic acid and ^{125}I were separated on Sephadex G50. The nucleic acid peak was dialysed exhaustively against SSC buffer. The specific activity obtained was 6×10^4 counts per minute (cpm) per μg nucleic acid for measles RNA, 3×10^5 for SV–40 DNA, 3×10^4 for rubella RNA, and 6×10^4 for RD–114 RNA.

FILTER HYBRIDISATION

Essentially the method of Gillespie and Spieglemann (1965) was used in which the cellular nucleic acid derived from the synovial membranes, synovial fluid cells, peripheral blood lymphocytes, synovial fibroblasts, and various cell lines plus calf thymus DNA were immobilised on Millipore nitrocellulose filter discs. The filters each contained 20 μg nucleic acid. Hybridisation was carried out in small sealed vials containing iodinated viral nucleic acid (in the case of viral DNA it was preheated for 15 min at 80°C), 20 μl 5% SDS, and 4 μg cold carrier DNA (calf thymus DNA, except for calf thymus control vial in which 4 μg human DNA was used as carrier), and 3 × SSC to make the final volume 1 ml. The vials contained up to 5 filters with nucleic acid plus 2 blanks. Incubation was carried out at 62°C for 22 hours. The filters were washed in 3 × SSC with several changes of buffer, digested with S_1 nuclease (Biochemicals Inc.) to remove unhybridised nucleic acid, and rewashed. They were counted in a Wallace-LKB gamma counter.

IN-SITU HYBRIDISATION

The method of Pardue and Gall (1975) was followed in outline. Frozen sections were prepared of synovial membranes, and various cell lines were grown on glass slides until semiconfluent. Two methods of fixation were used: (1) 0·1% Glutaraldehyde at 4°C for 30 min followed by dehydration in 50%, 70%, 90%, and 100% ethanol. (2) Acetic acid: ethanol, 1:3 for 2 min, followed by dehydration in 95% ethanol. These slides were treated with 200 μl pancreatic RNase (100 μg/ml in 2 × SSC, Sigma) for 1 hour at 37°C, washed in 2 × SSC, then dehydrated again. They were denatured in 0·07N NaOH for 2–3 min and dehydrated.

With all slides 10 μl hybridisation mix was added which consisted of 100 μl 42% formamide, 20 μl 10X acetate buffer (3M NaCl, 0·1 M Na acetate, pH 5), and 80 μl iodinated viral nucleic acid (about 25 000 cpm) or SSC (control). Hybridisation was carried out at 46°C for 22 hours under cover-slips sealed with Cowgum. The slides were washed in acetate buffer, treated with S_1 nuclease for 1 hour at 37°C, rewashed, dehydrated in ethanol, and covered with Ilford K2 emulsion. They were developed with Kodak D 19 after periods of 4 to 8 weeks.

Results

SV–40

1·7 μg (500 000 cpm) iodinated SV–40 DNA was used in hybridisation with nitrocellulose filters containing 20 μg DNA from calf thymus, from peripheral blood lymphocytes of 4 normal people, from 2 rheumatoid synovial membranes, and from 2 rheumatoid synovial fibroblast strains. As a positive control for the method DNA was extracted from 2 rheumatoid synovial strains and 1 non-rheumatoid strain transformed by SV–40. These should contain an integrated copy (or copies) of the viral genome. The cpm of the filters after treatment with S_1 nuclease are shown in Table 1. These represent an average of 2 to 4 determinations done within 4 weeks of iodinating SV–40.

It can be seen that the cpm of the filters containing DNA from the SV–40 transformed synovial cells are 2 to 3 times more than the other synovial preparations. The latter were considered therefore not to contain SV–40 viral sequences.

MEASLES

0·45 μg (27 000 cpm) and 0·045 μg (2700 cpm) iodinated measles RNA were used in hybridisation with nitrocellulose filters containing 20 μg DNA from calf thymus, from 3 synovial membranes of RA

Table 1 *Hybridisation of SV–40 ^{125}I–DNA with cellular DNA*

	cpm per filter
No nucleic acid	238
Calf thymus DNA	275
Peripheral blood lymphocytes, 2	199
Peripheral blood lymphocytes, 3	194
Peripheral blood lymphocytes, 4	188
Peripheral blood lymphocytes, 5	186
Rheumatoid synovial membrane, 1	220
Rheumatoid synovial membrane, 8	202
Rheumatoid synovial fibroblast strain, 2	263
Rheumatoid synovial fibroblast strain, 7	222
SV–40 transformed rheumatoid synovial fibroblast strain, 5	442
SV–40 transformed rheumatoid synovial fibroblast strain, 7	408
SV–40 transformed non-rheumatoid synovial fibroblast strain, 10	666

patients and 1 juvenile RA, from 1 synovial membrane of an OA patient, and from 1 synovial fluid from an RA patient. The results can be seen in Table 2; they represent an average of 2 determinations. The counts obtained were all lower than the filter with no nucleic acid or with DNA from calf thymus, and it was concluded that no hybridisation had taken place.

Moreover, in-situ hybridisation was carried out on frozen sections cut from 4 synovial membranes. These came from 2 patients with RA and 1 each with OA and a popliteal cyst. The sections were all fixed in acetic acid/ethanol and treated with RNase before denaturing the DNA. After 4 and 8 weeks the slides were developed and did not contain more grains over the cells than the control slides.

These hybridisations were set up to detect measles-specific DNA in synovial cells, and no positive control was included for this system. Some evidence has been produced to suggest that measles in a chronic type of infection of chick embryo fibroblasts goes through a DNA proviral stage (Zhdanov, 1975), but this has not been substantiated subsequently by Holland *et al.* (1976) using hybridisation techniques and transfection.

RUBELLA

2 μg (60 000 cpm) and 0·2 μg (6000 cpm) iodinated rubella RNA were used in hybridisation with DNA and RNA from 10 synovial sources as listed in Table 3. No sample was considered to show hybridisation. DNA and RNA were extracted from BHK–21 cells lytically infected with rubella and from LLC–MK_2 cells chronically infected with rubella to act as controls for the system. These results are shown in Table 4. It may be seen that there are higher counts associated with RNA from both the chronically infected and lytically infected cells as compared to the RNA form the uninfected cells, while the DNA does not show this. This is perhaps surprising as rubella is a positive strand RNA virus, and one would imagine only small quantities of the negative strand being present in the infected cells at any one time. Also from this there is no evidence for a DNA intermediate in the replication, even in a chronic infection, as has been postulated recently by Sato *et al.* (1977) Work is continuing on this chronically infected cell line and will be published shortly (Norval, 1978, to be published).

In-situ hybridisation was also carried out with

Table 2 *Hybridisation of measles ^{125}I–RNA with cellular nucleic acid*

Nucleic acid	*Specimen*	*Diagnosis*	*cpm per filter*	
			0·45 μg ^{125}I–RNA	*0·045 μg ^{125}I–RNA*
None			1906	303
DNA	Calf thymus		2570	373
DNA	Synovial membrane A	Rheumatoid arthritis	1683	258
DNA	Synovial membrane B	Rheumatoid arthritis	1610	234
DNA	Synovial membrane C	Rheumatoid arthritis	1467	280
DNA	Synovial membrane D	Juvenile rheumatoid arthritis	—	257
DNA	Synovial membrane E	Osteoarthrosis	1960	—
DNA	Synovial fluid F	Rheumatoid arthritis	2065	272

A dash means not done.

Table 3 *Hybridisation of rubella virus ^{125}I–RNA with cellular nucleic acid*

Nucleic acid	*Specimen*	*Diagnosis*	*cpm per filter*	
			2 μg ^{125}I–RNA	*0·2 μg ^{125}I–RNA*
None			3035	315
DNA	Calf thymus		4644	438
DNA	Synovial membrane A	Rheumatoid arthritis	2428	283
DNA	Synovial membrane B	Rheumatoid arthritis	2086	261
DNA	Synovial membrane C	Rheumatoid arthritis	3192	360
DNA	Synovial membrane E	Osteoarthrosis	3225	291
DNA	Synovial fluid F	Rheumatoid arthritis	3530	350
RNA	Synovial membrane G	Rheumatoid arthritis	—	386
RNA	Synovial fluid H	Reiter's disease	—	404
RNA	Synovial fluid I	Polyarthritis after rubella	—	216
RNA	Synovial fibroblast strain J derived from synovial membrane	Osteoarthrosis	3626	—
RNA	Synovial fibroblast strain K derived from synovial membrane	Rheumatoid arthritis	2979	—

A dash means not done.

Table 4 *Hybridisation of rubella ^{125}I–RNA with cellular nucleic acid*

	cpm per filter		
	2μg ^{125}I–RNA	*0·4 μg ^{125}I–RNA*	*0·2 μg ^{125}I–RNA*
No nucleic acid	2168	476	347
DNA from BHK cells	2425	462	265
DNA from BHK cells lytically infected with rubella virus	2005	495	283
RNA from BHK cells	2709	630	394
RNA from BHK cells lytically infected with rubella virus	4242	1065	467
DNA from LLC–MK_2 cells	1586	456	281
DNA from LLC–MK_2 cells chronically infected with rubella virus	1313	410	371
RNA from LLC–MK_2 cells	2075	474	347
RNA from LLC–MK_2 cells chronically infected with rubella virus	2961	797	538

synovial membrane sections from the same patients as in the measles experiments above, except that these were fixed and pretreated in 2 ways, firstly, to degrade RNA and denature DNA, and, secondly, to leave the RNA intact. They were developed after 4 and 8 weeks and showed no more grains over the cells than the control slides. In a similar manner LLC–MK_2 cells and LLC–MK_2 cells chronically infected with rubella were grown on glass slides and fixed by the 2 methods. The ones treated with RNase and denatured did not contain more grains after an 8-week exposure than the control slides. The results for the set fixed in glutaraldehyde are shown in Table 5.

It may be seen that there are more grains per cell in the LLC–MK_2 cells chronically infected with rubella virus and hybridised with ^{125}I-rubella RNA than in the uninfected cells. Thus it may be assumed that the synovial cells contain either no rubella nucleic acid or less than is synthesised in the chronically infected system used here as a control for the method.

RD–114

As with the other viral nucleic acids, 1·5 μg (40 000 cpm) iodinated RD–114 RNA was used in filter hybridisation with DNA from various synovial sources. The results are shown in Table 6, and each count represents an average of 3 determinations. In addition DNA extracted from RD cells and RD infected with RD–114 were used, and the results given in Table 6. None of the filters containing nucleic acid from synovial cells was thought to show hybridisation to the viral probe, but the counts on the RD–114 DNA filter were about twice that of the RD filter, demonstrating that the RD cells infected with RD–114 contained viral sequence(s) in the DNA.

Table 5 *Hybridisation of rubella ^{125}I–RNA in situ*

	Average number of grains per cell (20 cells counted)
LLC–MK_2 cells hybridised with SSC buffer	9
LLC–MK_2 cells hybridised with ^{125}I–rubella virus RNA	8
LLC–MK_2 cells chronically infected with rubella virus and hybridised with SSC buffer	14
LLC–MK_2 cells chronically infected with rubella virus and hybridised with ^{125}I-rubella virus RNA	46

Discussion

Molecular hybridisation has been widely used as a tool in recent years to probe for viral nucleic acid sequences in human tissues, especially in various malignancies such as lymphomas (Hehlmann *et al.*, 1972) and leukaemias (Larsen, *et al.*, 1975). These experiments are difficult to do satisfactorily, being dependent, among other things, on obtaining nucleic acid preparations that are pure enough plus good control material, and on labelling the viral nucleic acid sufficiently. Despite these inherent difficulties it seemed worthwhile in view of the negative results from virus isolations and in screening for viral antigens on synovial cells to look for viral nucleic acid sequences in rheumatoid material.

The method used here was a simple one which consisted of immobilising the denatured cellular

Table 6 *Hybridisation of RD–114 ^{125}I–RNA to cellular DNA*

Nucleic acid	*Specimen*	*Diagnosis*	*cpm per filter*
None			492
DNA	Synovial membrane B	Rheumatoid arthritis	349
DNA	Synovial membrane C	Rheumatoid arthritis	346
DNA	Synovial membrane L	Rheumatoid arthritis	493
DNA	Synovial fluid F	Rheumatoid arthritis	478
DNA	Synovial fluid M	Rheumatoid arthritis	348
DNA	Synovial fluid N	Inflammatory polyarthritis	489
DNA	RD cells		473
DNA	RD cells infected with RD–114		835

DNA on nitrocellulose filter discs and then hybridising in solution with iodinated viral nucleic acids from 4 viruses—SV–40, measles, rubella, and RD–114. Owing to the relative scarcity of clinical material, especially of non-RA control material, the kinetics of hybridisation were not measured. A comparison was made between the counts on filters containing nucleic acid from RA patients with those from non-RA patients at one time. In addition, the technique of iodination should give ideally a specific activity of around 10^7 cpm/μg nucleic acid but, here, only about 10^5 was achieved, and this will obviously lead to a distinct loss in sensitivity.

However, the control systems used, which were synovial fibroblasts transformed with SV–40, LLC–MK_2 cells chronically infected with rubella virus and RD cells infected with RD–114 virus, were all positive by the methods used. Various alternative methods of labelling the viral nucleic acid were tried. These included adding ^{3}H-uridine during measles infection of Vero cells, but the specific activity of the extracted nucleic acid was not as high as with iodination. Further, a ^{3}H-cDNA was made to RD–114 by the method of Rothenberg and Baltimore (1976), but again the extracted and purified cDNA was not labelled sufficiently. It would have been preferable to use cDNA, as it will hybridise to viral mRNA in cells containing the viral genome, and in most cases this will amplify the viral information within the cells and allow easier detection by hybridisation.

Thus, despite these reservations about the hybridisation assay, it proved sufficiently sensitive to detect viral sequences in the control cells. The negative results obtained with the rheumatoid cells are valid at this level of sensitivity, that is, they would have to contain at least the same number of genome equivalents as in the positive control systems. If, on the other hand, only a few cells from the population of synovial cells contain viral information, such as a certain class of lymphocytes perhaps, then this would probably not be detectable in the assay system used. Nucleic acids were extracted from synovial membranes, which consist of a mixed population of cells, mostly synovial cells lining with some lymphocytes, polymorphs, monocytes, and macrophages, and from synovial fluids, which again consist of a mixture of cells, largely polymorphs with some lymphocytes, monocytes, macrophages, and a few lining cells. As these specimens came directly from the patient without prior culturing, it was hoped that the total mass would contain the relevant population of cells. All the nucleic acids extracted from these different sources, however, showed no homology with the viral probes prepared from SV–40, measles, rubella, and RD–114.

Another method tried for measles and rubella probes, in addition to filter hybridisation, was in-situ hybridisation, but, again, no homology was found with the nucleic acids in synovial membrane sections from RA patients and patients with other conditions. This technique has been used successfully to detect viral nucleic acids in several systems, such as viral mRNA in adeno infected and transformed cells (Moar and Jones, 1975). One clinical situation of interest in this context is that of detecting viral DNA in sheep with visna (Haase *et al.*, 1977). Sections of choroid plexus were taken, and, while only 0·025% cells showed the presence of membrane p30 antigen by immunofluorescence, 18% contained viral DNA by in-situ hybridisation using ^{3}H–cDNA of visna virus as probe. It was calculated from the number of grains over the nucleus that there were 100–200 copies of the viral genome per cell, which is a high level of gene reiteration and very helpful, obviously, in detection.

The 4 viruses used here as probes were selected for various reasons. SV–40 can be a transforming virus, and other closely related members of the same genus, *Polyomavirus*, have been described in human beings, especially when immunosuppressed. RD–114 is an endogenous cat retrovirus which has a very close relationship to the baboon endogenous virus, and it was hoped that it would also show some homology with a putative human retrovirus. In systemic lupus erythematosus (SLE) an antigen cross-reacting to a retrovirus, SP104, has been described on blood lymphocytes of human and canine cases (Schwartz, 1975). SP104 was first produced by mouse tumour cells in culture after the tumour was induced by a cell free filtrate of canine SLE spleen. However, very recently Quimby *et al.* (1978) have reported that there is no homology using techniques of competitive hybridisation between the cDNA of this virus and RNA from cells of dogs and human beings with SLE. Measles was chosen because of its association with subacute sclerosing panencephalitis in man, a slow chronic progressive condition (Hall and ter Meulen, 1976), and also structures like paramyxovirus nucleocapsids have been described in synovial lymphoreticular cells in rheumatoid arthritis (Neumark and Farkas, 1973). Finally rubella was selected for the reasons outlined in Hart and Marmion (1977) and McCormick *et al.* (1978).

We are continuing this study by using adeno–2 DNA as a probe and looking at in-situ hybridisation to RNA in sections of synovial membranes and synovial fibroblasts grown on slides. Also the kinetics of hybridisation to RNA extracted from rheumatoid membranes and from rheumatoid and non-rheumatoid synovial fibroblasts are being examined.

We thank Professor B. R. Marmion and Dr D. Hamerman for very helpful discussion, and the Nuffield Foundation and the US Public Health Service Research Grant No. AM 15796 for grant support.

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J. gen. Virol. (1979), 43, 289–298
Printed in Great Britain

Mechanism of Persistence of Rubella Virus in LLC-MK$_2$ Cells

By MARY NORVAL

Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

(*Accepted* 8 *November* 1978)

SUMMARY

LLC-MK$_2$ cells chronically infected with two strains of rubella virus, HPV-77 and Thomas, have been examined over several months to find out the mechanism of persistence. Evidence is given for the presence of defective particles in these cultures by finding virion RNA which sedimented at 12S instead of the 40S typical of the fully infectious virus. A 'provirus' DNA copy of the rubella virus genome was not detected by methods which included filter hybridization and *in situ* hybridization, or by treatment of the chronically infected cells with mitomycin C, actinomycin D or 5-bromodeoxyuridine. In addition, the chronically infected cells contained RNA-dependent RNA polymerase activity, but no RNA-dependent DNA polymerase activity.

INTRODUCTION

Many animal viruses seem to persist in natural infections and in various cell cultures *in vitro* and the mechanism by which this occurs has been the subject of some research in the past few years, having obvious implications in 'inapparent' infections, slow virus diseases and latency. Not much is known about how the genome of RNA animal viruses persist, apart from retroviruses, but several cell cultures have been established which are chronically infected with various RNA viruses and these should give some insight into the mechanism.

Evidence has been gathered, on the one hand, to indicate that a proviral DNA form may be important. The work of Furman & Hallum (1973) and Preble & Youngner (1973) on mouse L cells persistently infected with a temperature sensitive (*ts*) mutant to Newcastle disease virus and that of Simpson & Iinuma (1975) on bovine embryonic kidney cells persistently infected with a *ts* mutant of respiratory syncytial virus, may be cited as examples. Zhdanov (1975) described similar results in three systems of chronic infection in which low titres of infectious virus were being produced. Finally Sato *et al.* (1976) have established a cell line, BHK 21/WI-2, persistently infected with rubella virus which releases a latent virus and a *ts* variant of rubella virus with reverse transcriptase activity. They subsequently provided evidence, from treatment of the cultures with various chemicals, that rubella virus may persist in a proviral form within these cells (Sato *et al.* 1977).

On the other hand, there is also evidence to suggest that proviral DNA may not be a necessary stage in persistent infection with RNA viruses. Thus Holland *et al.* (1976) found that BHK-21 cells persistently infected with vesicular stomatitis virus (VSV) and shedding low levels of infectious virus over 2 years did not contain proviral DNA by hybridization or by transfection. Similar results were described for other persistent infections with various negative-strand RNA viruses like measles, mumps, influenza, LCM and rabies. Defective interfering particles were found to be a major factor in establishment and maintenance of

0022-1317/79/0000-3416 $02.00

the chronically-infected state and temperature sensitivity of the shed virus was also important.

A line of LLC-MK_2 cells has been described which can be persistently infected with rubella virus and which grew for at least 50 subcultures with no loss of ability to liberate infectious virus (Maassab *et al.* 1964). A similar line was established in our laboratory several months ago (Hart & Marmion, 1977) and has been studied subsequently to try to find out how the virus persists in this situation.

METHODS

The conditions used to initiate and maintain a persistent infection of LLC-MK_2 cells (American Cell Culture Type Collection) with HPV-77 rubella virus, vaccine strain and an isolate Thomas have been described previously (Hart & Marmion, 1977). The presence of rubella virus was confirmed by indirect immunofluorescence (Lennette *et al.* 1967) and by labelling with ^{3}H-uridine as outlined below. Preparation of antisera to HPV-77 and indirect immunofluorescence techniques have also been described by Hart & Marmion (1977).

In addition, for lytic infections, BHK-21 or Vero cells were used. They were grown in Earle's based Eagle's medium containing penicillin and streptomycin plus 2 or 5 % foetal calf serum.

Curing of cells was performed as outlined by Holland *et al.* (1976) and growth in semi-solid agar as described by Macpherson & Montagnier (1964).

Purification of rubella virus and characterization of virus RNA. Rubella virus from Vero cells infected 4 days previously and from chronically infected LLC-MK_2 cells, was labelled with ^{3}H-uridine (2·5 μCi/ml, Radiochemical Centre, Amersham) for 24 h. The culture supernatant was clarified at 10000 *g* for 10 min and then either precipitated with an equal vol. of cold saturated $(NH_4)_2SO_4$ or centrifuged at 30000 rev/min for 1 h in an SW40 rotor. The deposit was suspended in a small vol. of buffer, 0·01 M-tris-HCl, 1 mM-EDTA, pH 7·1 (Grayzel, 1973). It was layered on to sucrose density gradients (20 to 70 %) and centrifuged at 25000 rev/min for 18 h in an SW40 rotor. Fractions were collected from the base of the tube, precipitated with cold 10 % TCA and counted after filtration through glass fibre discs (Norval & Marmion, 1976). Under these conditions rubella virus has a density of 1·19 g/ml.

To characterize rubella virus RNA, the band of density 1·18 to 1·20 g/ml was taken from the sucrose density gradients and the virus pelleted. RNA was extracted and characterized by the methods outlined in Hovi & Vaheri (1970).

Treatment with chemicals. The chronically infected cells were cultured in medium containing the following substances, and then labelled with ^{3}H-uridine for 24 h and separated on sucrose density gradients as described above to establish the quantity of rubella virus being released: (1) BrdUrd (Sigma), 20 μg/ml for 3 days; (2) actinomycin D (Boehringer), 0·1 μg/ml for 24 h or 5 μg/ml for 3 h followed by incubation for 1 or 3 days in medium without actinomycin D; (3) mitomycin C (Sigma), 0·5 μg/ml for 7 h, followed by incubation for 3 days in medium without mitomycin C; (4) poly(I).poly(C) (Biochemicals Inc.), 10 μg/ml for 1 h.

Extraction of cellular RNA and DNA. DNA and RNA were extracted from BHK cells, 3 days after infection with rubella virus, from uninfected BHK cells, from chronically infected LLC-MK_2 cells and from uninfected LLC-MK_2 cells using the method of Simpson & Iinuma (1975). The precipitated nucleic acids after chloroform/isoamyl alcohol extraction were treated with RNase (50 μg/ml, pre-treated at 80 °C for 10 min, Sigma) for DNA preparations, or with DNase (20 μg/ml, Sigma) for RNA preparations. The final pre-

cipitation was with ethanol and the nucleic acid was dissolved in SSC buffer (0·15 M-NaCl, 0·015 M-sodium citrate). The ratio of absorbance at 260 to 280 nm was at least 1·85, and generally around 10^8 cells were used for extraction. The nucleic acid was stored at −70 °C until use.

Preparation of virus nucleic acid and iodination. Rubella virus was grown in Vero cells in roller bottles. It was purified by banding on sucrose density gradients as described above. RNA was extracted from the concentrated virus preparation. The vol. was made up to 10 ml in TNES buffer (0·1 M-tris, pH 9·0, 0·1 M-NaCl, 1 mM-EDTA and 0·1 % SDS) and extracted three times with TNE (0·1 M-tris, pH 9·0, 0·1 M-NaCl, 1 mM-EDTA)-saturated phenol containing 10 % chloroform. The virus nucleic acid was precipitated with ethanol and the final precipitate dissolved in 0·3 ml sterile SSC.

Rubella RNA (200 μg) was iodinated using a modification of the method of Commerford (1971). A mixture was made of 0·3 ml 0·5 M-sodium acetate buffer, pH 5·0; 0·3 ml SSC containing the rubella RNA; 0·3 ml thallic chloride, 2·5 mM in 0·1 M-acetate buffer, pH 5·0; and 0·3 ml potassium iodide, 0·125 mM, which had previously been added to 600 μCi ^{125}I. It was incubated for 1 h at 60 °C and the iodinated RNA separated on Sephadex G 50. The nucleic acid was dialysed exhaustively against SSC buffer and stored at −70 °C. The sp. act. obtained was 3×10^4 ct/min/μg nucleic acid.

Filter hybridization. Essentially the method of Gillespie & Spiegelman (1965) was used where the cellular nucleic acid was immobilized on Millipore nitrocellulose filter discs. The filters each contained 20 μg nucleic acid. Hybridization was carried out in small sealed vials containing iodinated rubella RNA, 20 μl 5 % SDS, 4 μg cold carrier calf thymus DNA and 3 × SSC to make the final vol. 1 ml. The vials contained up to five filters with nucleic acid plus two blanks. Incubation was carried out at 62 °C for 22 h. The filters were washed in 3 × SSC with several changes of buffer, digested with S_1 nuclease (Biochemicals Inc.) to remove unhybridized nucleic acid and re-washed. They were counted in a Wallac-LKB gamma counter.

In situ *hybridization.* The method of Pardue & Gall (1975) was followed in outline. Chronically infected LLC-MK_2 cells and uninfected cells were grown on glass slides until semi-confluent. Two methods of fixation were used. (*a*) 0·1 % glutaraldehyde at 4 °C for 30 min, followed by dehydration in 50, 70, 90 and 100 % ethanol. (*b*) Acetic acid:ethanol, 1:3, for 2 min, followed by dehydration in 95 % ethanol. These slides were treated with 200 μl pancreatic RNase (100 μg/ml, in 2 × SSC, Sigma) for 1 h at 37 °C, washed in 2 × SSC, then dehydrated again. They were denatured in 0·07 M-NaOH for 2 to 3 min, then dehydrated.

With all slides, 10 μl hybridization mix was added which consisted of 100 μl 42 % formamide, 20 μl 10 × acetate buffer (3 M-NaCl, 0·1 M-Na acetate, pH 5·0) and 80 μl rubella ^{125}I-RNA or SSC (control). Hybridization was carried out at 46 °C for 22 h under coverslips sealed with cowgum. The slides were washed in acetate buffer, treated with S_1 nuclease for 1 h at 37 °C, re-washed, dehydrated in ethanol and covered with Ilford K2 emulsion. They were developed using Kodak D 19 after periods of 4 to 8 weeks.

Polymerase assays

RNA-dependent RNA polymerase. Vero cells lytically infected with rubella virus 5 days previously, chronically infected LLC-MK_2 cells and uninfected cells were harvested with glass beads, washed in cold 0·1 M-tris buffer, pH 8·0, and lysed with Nonidet P-40, added at a final concentration of 0·1 %. The nuclei were removed by spinning at 2000 *g* for 15 min at 4 °C and the supernatant assayed for RNA-dependent RNA polymerase activity using a modification of the method of Scholtissek & Rott (1969). Assay mixtures contained

1 μmol each of ATP, GTP and CTP, 3·6 μmol $MgCl_2$, 4·5 μmol dithiothreitol, 30 μmol tris buffer, pH 8·0, and 2·5 μCi ^{3}H-UTP (10 to 30 Ci/mol, Radiochemical Centre, Amersham) and cell lysate in a total vol. of 200 μl. Incubation was carried out at 37 °C and samples of 40 μl were spotted on filter paper discs at intervals of 15 min. Filters were fixed in cold 10 % trichloroacetic acid (TCA), washed five times in cold 10 % TCA before being air-dried and counted in PPO-toluene scintillator in a Packard Tri-Carb liquid scintillation counter. Protein content was estimated by the method of Lowry *et al.* (1951).

RNA-dependent DNA polymerase. Culture supernatants from Vero cells, lytically infected with rubella virus 5 days previously, and chronically infected LLC-MK_2 cells were precipitated by polyethylene glycol (Bronson *et al.* 1975). They were assayed for RNA-dependent DNA polymerase (reverse transcriptase) activity using the method of Lieber *et al.* (1973), whereby the incorporation of ^{3}H-methyl TTP (25 Ci/μmol, Radiochemical Centre, Amersham) into acid-insoluble material was measured in the presence of the artificial template/primer polyadenylic acid decathymidylic acid (poly(dA).poly$(dT)_{10}$, Boehringer Corp.). Protein content was estimated by the method of Lowry *et al.* (1951).

RESULTS

Persistent infection of LLC-MK_2 cells

Persistent infection of LLC-MK_2 cells with either HPV-77 or Thomas strains of rubella virus could be achieved quite easily by infecting the cells and leaving them for a period of 3 weeks before subculturing. Only a few cells survived at this stage but they gradually grew until they formed patchy cell sheets. The cells divided more slowly than the uninfected cells thereafter and were irregular in shape and size. They consistently liberated small amounts of virus over at least 40 subcultures and a period of 2 years. The presence of rubella was detected in two ways, either by labelling with ^{3}H-uridine or by indirect immunofluorescence.

A typical result from labelling a chronically infected LLC-MK_2 culture is shown in Fig. 1 and a very similar picture was obtained from labelling Vero cells 4 days after infection with rubella virus. McCombs & Rawls (1968) found that the density of rubella was 1·19 g/ml and it may be seen that there was a peak of incorporation into acid-insoluble material at this density with a spread on either side. The amount varied with the number of days after trypsinization, most being liberated within 2 days of subculturing or after recovery from storage in liquid nitrogen, but the quantity did not decrease much with passage over the 2 year period.

A description of the immunofluorescent results has already been given by Hart & Marmion (1977). Briefly, acetone-fixed cells chronically infected with rubella showed a granular cytoplasmic staining which was apparent at early subcultures in about 80 % of the cells. This percentage declined with passage to about 5 % at the 36th subculture, in the case of cells chronically infected with HPV strain, and to about 50 % at the 15th subculture of cells chronically infected with Thomas strain. There was only weak, or no, membrane staining when using unfixed chronically infected cells, while BHK cells lytically infected with rubella showed both cytoplasmic and membrane staining.

Virus RNA

Culture supernatants from Vero cells infected 3 days previously with rubella virus and from chronically infected LLC-MK_2 cells (120 and 240 ml respectively) were harvested after labelling with ^{3}H-uridine. The virus was purified on sucrose density gradients and pelleted. It was lysed in a buffer containing SDS and the virus RNA characterized on a 15 to 30 % sucrose density gradient as outlined by Hovi & Vaheri (1970). The result may be seen in Fig. 2. An 18S marker of mouse ribosomal RNA, kindly supplied by Dr Keith

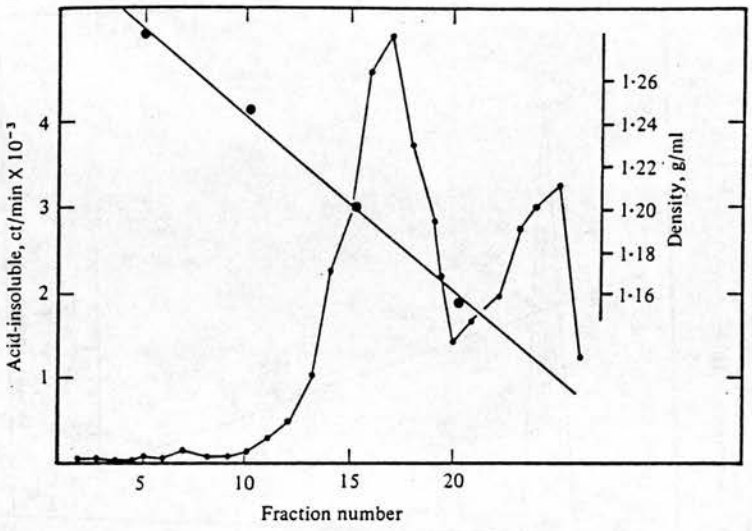

Fig. 1. Acid-insoluble ct/min in sucrose density gradient fractions from culture supernatants of LLC-MK_2 cells persistently infected with rubella virus HPV-77 strain and labelled with ^{3}H-uridine.

James, was run at the same time. The sedimentation coefficients were calculated using the tables of McEwan (1967). RNA from rubella produced by lytically infected Vero cells was of two sizes, 40S and around 12S, in the ratio of about 2:1. The RNA from rubella produced by chronically infected LLC-MK_2 cells, was also of two sizes 40S and around 12S, with the ratio this time being about 1:4.

Growth at different temperatures and cloning

The results of Preble & Youngner (1973), Simpson & Iinuma (1975) and Sato *et al.* (1976) indicate that *ts* variants of NDV, RSV and rubella virus are important in persistent infections. Thus when LLC-MK_2 cells chronically infected with Thomas strain were cultured at 43, 40 and 31·5 °C they did not survive at 43 °C. At 40 °C the cells became rather rounded and did not remain attached to the glass surface after a period of 3 days. Cells were also cultured at 31·5 °C for 3 weeks during which they had a normal appearance although they grew much more slowly and were more spaced out than at 37 °C. They were subcultured on to coverslips which were subsequently examined by indirect immunofluorescence. The percentage which contained rubella antigen was not noticeably different from cells maintained under the same conditions at 37 °C.

Holland *et al.* (1976) have observed curing of BHK-21 cells persistently infected with VSV by cloning or by prolonged antibody treatment of a lightly seeded monolayer. The former was tried using 60 mm Petri dishes seeded with 250, 500 and 1000 LLC-MK_2 cells chronically infected with either HPV-77 or Thomas strains which were then left for 6 weeks at 37 °C with weekly changes of medium. At the highest cell concentration, three clones grew on different plates which were examined by indirect immunofluorescence after subculturing to coverslips. In all cases the cells showed the same staining pattern as the chronically infected LLC-MK_2 cells with no difference in the percentage of cells infected as compared to that observed before cloning. In addition, an attempt was made to clone the cells in semi-solid agar where 10^3, 10^4 or 10^5 LLC-MK_2 cells chronically infected with HPV-77 at subculture 2 and 43 were placed in Petri dishes. These were observed over a 6 week incubation period but no colonies were seen in any plate. Occasionally clumps were found but these consisted of less than 6 cells.

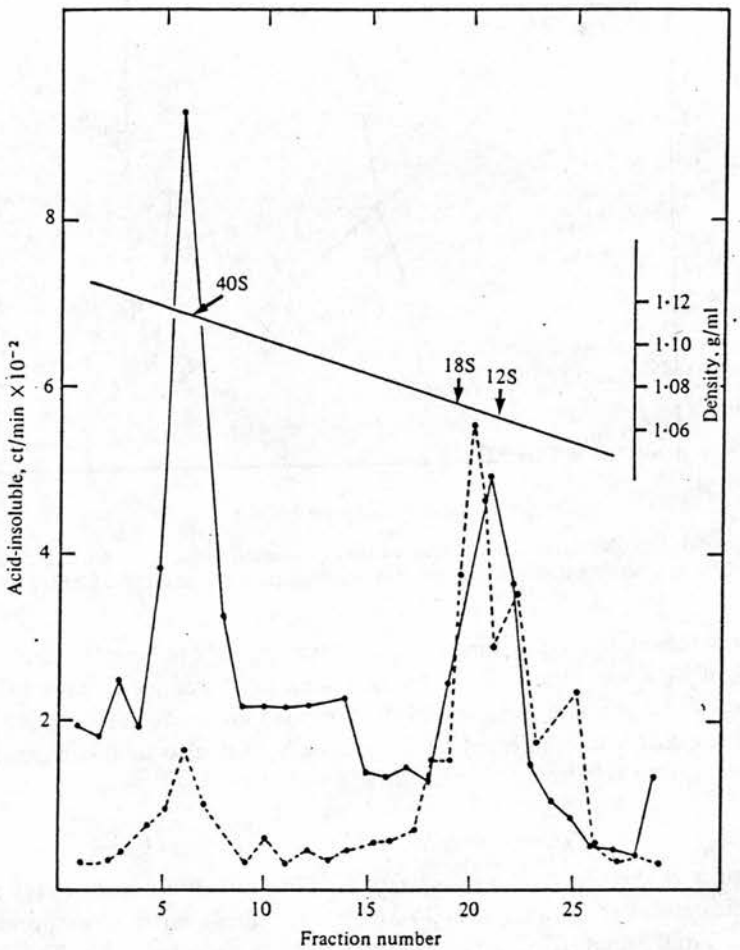

Fig. 2. Acid-insoluble ct/min in sucrose density gradient fractions of rubella virus RNA purified from culture supernatants of LLC-MK_2 cells chronically infected with rubella virus (●---●) and Vero cells lytically infected with rubella virus (●——●) and labelled with ^{3}H-uridine.

Effect of various chemicals

Following the methods of Sato *et al.* (1976), LLC-MK_2 cells persistently infected with HPV-77 or Thomas strains were treated with actinomycin D (5 μg/ml) for 3 h. The cultures were immediately labelled with ^{3}H-uridine or were left for 1 or 3 days before labelling. It was found that this level of actinomycin D was quite toxic causing the death of almost all cells in 3 days. Labelling immediately after actinomycin D treatment indicated no decrease in the acid-insoluble counts at density 1·19 but the viability of the cells decreased by 50 % over this period, thus making it difficult to quantify this experiment. Actinomycin D added at a concentration of 0·1 μg/ml (Woods & Robbins, 1968) followed by labelling with ^{3}H-uridine had no effect on the release of rubella virus.

Treatment with BrdUrd enhanced rubella virus production by twofold as measured by the quantity of acid-insoluble counts at density 1·19, while mitomycin C seemed to have little or no effect. These results are shown in Fig. 3. Poly(I).poly(C) treatment of the infected cells, which should induce interferon production, reduced the quantity of labelling at density 1·19 by 50 %.

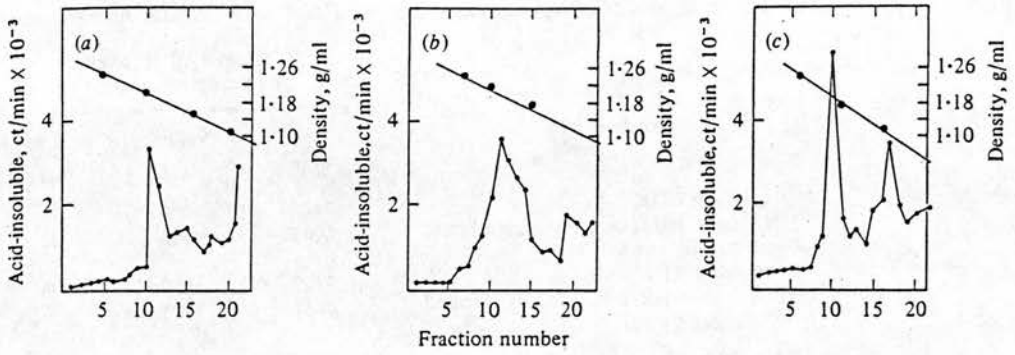

Fig. 3. Acid-insoluble ct/min in sucrose density gradient fractions from culture supernatants of LLC-MK_2 cells persistently infected with rubella virus HPV-77 strain and labelled with ^{3}H-uridine: (*a*) control, (*b*) treated with mitomycin C, or (*c*) treated with BrdUrd.

Table 1. *Polymerase activities in rubella infected cells*

Cells	RNA-dependent RNA polymerase activity* of cell lysates	RNA-dependent DNA polymerase activity† of culture supernatants
Lytically infected Vero	10·7	0
Uninfected Vero	0	0
Chronically infected LLC-MK_2	8·0	0
Uninfected LLC-MK_2	0	0

* Activity measured as increase in incorporation (ct/min) of ^{3}H-UTP into acid-insoluble material in 1 h per μg protein.

† Activity measured as increase in incorporation (ct/min) of ^{3}H-TTP into acid-insoluble material in 1 h per μg protein.

Polymerase activity

RNA-dependent RNA polymerase is present in negative-strand RNA viruses and is synthesized in infected cells by positive-strand RNA viruses, while RNA-dependent DNA polymerase is associated with retroviruses and their replication. To obtain some information about the transcription of rubella virus RNA in the chronic and lytic states, infected cells were lysed and assayed for RNA-dependent RNA polymerase and the culture supernatants, after concentration, were assayed for RNA-dependent DNA polymerase. As controls the uninfected LLC-MK_2 and Vero cultures were used. The results are shown in Table 1. There was no RNA-dependent DNA polymerase activity demonstrable in any sample, while both the chronically infected LLC-MK_2 cells and the lytically infected Vero cells showed RNA-dependent RNA polymerase activity.

Hybridization

Holland *et al.* (1976) have studied BHK cells persistently infected with VSV and, by hybridization in solution of a ^{3}H-cDNA of VSV RNA to cellular DNA, were unable to detect virus DNA. The sensitivity was calculated to be at the level of 1 virus genome per 40 cells or 1/40th virus genome per cell.

In the present work RNA and DNA were extracted from BHK-21 cells lytically infected with rubella virus, from LLC-MK_2 cells chronically infected with rubella virus and from

Table 2. *Hybridization of rubella virus ^{125}I-RNA with cellular nucleic acid*

Nucleic acid	^{125}I (ct/min/filter; average of 2 determinations) 2 μg	0·4 μg	0·2 μg
None	2168	476	347
DNA from BHK cells	2425	462	265
DNA from BHK cells lytically infected with rubella virus	2005	495	283
RNA from BHK cells	2709	630	394
RNA from BHK cells lytically infected with rubella virus	4242	1065	467
DNA from LLC-MK$_2$ cells	1586	456	281
DNA from LLC-MK$_2$ chronically infected with rubella virus	1313	410	371
RNA from LLC-MK$_2$ cells	2075	474	347
RNA from LLC-MK$_2$ chronically infected with rubella virus	2961	797	538

Table 3. *Results of* in situ *hybridization of rubella virus ^{125}I-RNA to LLC-MK$_2$ cells chronically infected with rubella virus after an* 8 *week exposure*

	Average number of grains per cell (20 cells counted) Hybridization with SSC buffer (control)	Hybridization with ^{125}I-RNA of rubella virus
LLC-MK$_2$ cells	9	8
LLC-MK$_2$ cells chronically infected with rubella virus	14	46

their uninfected control cells. Two μg (60000 ct/min), 0·4 μg (12000 ct/min) and 0·2 μg (6000 ct/min) iodinated rubella virus RNA were used in hybridization with filters to which had been attached 20 μg cellular DNA or RNA. The results may be seen in Table 2. Each count represents an average of two determinations which were found to be very consistent. It may be seen that there are higher counts associated with RNA from both the chronically and lytically infected cells as compared to the RNA from the uninfected cells, while the DNA does not show this.

In situ hybridization was also carried out using LLC-MK$_2$ cells chronically infected with rubella virus and uninfected cells grown on glass slides and fixed in two ways, firstly to degrade RNA and denature DNA and secondly to leave the RNA intact. The slides were developed after 4 and 8 weeks. The first set where the RNA was degraded showed the same number of grains over the uninfected cells as the cells chronically infected with rubella. However, the result from the second set is shown in Table 3. There seemed to be a small amount of hybridization of rubella virus ^{125}I-RNA to the chronically infected LLC-MK$_2$ cells.

DISCUSSION

As already indicated in this paper, there have been various reports in the literature in the last few years outlining mechanisms whereby RNA viruses may persist and cause chronic infections in cell culture. Some systems investigated indicate that a DNA proviral form may exist like that described in the replication of retroviruses, while others do not

indicate such an intermediate and suggest that defective interfering particles may be important. Of particular interest in the present context is the work of Sato's group (Sato *et al.* 1976, 1977) on a persistent infection of BHK-21/WI-2 cells by rubella virus. Variants of the wild type virus were produced which were thought to be hybrids between rubella and the latent virus of BHK-21/WI-2 cells. They were temperature sensitive at 39·5 °C, possessed reverse transcriptase activity and could be induced by BrdUrd or mitomycin C, being blocked by actinomycin D. It was concluded that a DNA intermediate was involved in the replication of rubella virus in the persistently infected cells.

A similar investigation has been carried out here using LLC-MK_2 cells chronically infected with rubella virus and the evidence gathered does not implicate a DNA intermediate in this system. BrdUrd and mitomycin C had little or no effect on the quantity of virus released while actinomycin D affected the viability of the cells so badly that it could not be used at the concentration outlined by Sato *et al.* (1977). When actinomycin D was added at a much lower concentration, as used by Woods & Robbins (1968), there was no effect on the amount of virus released. These results do not support the involvement of a DNA intermediate in this chronic infection. Infectivity titres were not measured, the quantity of virus released being assayed by incorporation of ^{3}H-uridine into particles of density 1·19.

RNA polymerase assays of the infected cells showed RNA-dependent RNA polymerase activity in both the lytic and chronic infections. Virus purified from both types of infection did not show any RNA-dependent DNA polymerase activity. In addition, the results from hybridization did not indicate that a DNA form was involved in the chronic infection, although the specific activity of the probe, ^{125}I-rubella RNA, was not as high as desirable, hence reducing the sensitivity of the assay.

Attempts to cure the chronically infected cells by cloning, as have been described by Holland *et al.* (1976) for the VSV system, failed. However, results from characterizing the extracted virus RNA on sucrose density gradients were of interest as two peaks were found, one of 40S, which is the sedimentation coefficient described by Hovi & Vaheri (1970) for rubella virus RNA, and one much smaller, around 12S, which is presumably not infectious. This is present in a much larger proportion in the virus population released from the chronically infected cells, so that defective particles without the full complement of virus RNA may be present in considerable numbers in this situation.

It is not really possible to say whether a *ts* mutant is involved as virus release was not assayed by infectivity or plaquing at any stage but certainly the pattern of immunofluorescence observed in chronically infected cells did not change markedly between 31·5 and 37 °C and the infection did not become lytic.

The results would indicate no DNA intermediate but probably the presence of defective interfering particles as a major factor in the maintenance of chronic infection by rubella virus in LLC-MK_2 cells.

The skilful assistance of Miss Sheena Tuach is gratefully acknowledged, and the help of two Honours students, Miss Dorothy McLeod and Miss Lesley Penny. In addition I would like to thank Professor B. P. Marmion for many useful discussions.

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(*Received* 7 *August* 1978)

Annals of the Rheumatic Diseases, 1979, **38**, 507–513

Viruses and lymphocytes in rheumatoid arthritis. I. Studies on cultured rheumatoid lymphocytes

MARY NORVAL, HELENA HART, AND B. P. MARMION

From the Department of Bacteriology, University of Edinburgh Medical School, Edinburgh

SUMMARY Synovial fluid lymphocytes from patients with rheumatoid arthritis have been examined for evidence of a productive infection with retroviruses by electron microscopy, labelling with ^{3}H-uridine, growth in soft agar, and culturing in conditioned medium. No such viruses were detected. In addition, the synovial lymphocytes were activated before fusion and cocultivation with several cell lines which have proved permissive for primate retroviruses. Monitoring these cultures subsequently by reverse transcriptase assay, labelling with ^{3}H-uridine, and membrane immunofluorescence gave no indication that retroviruses were present.

A possible viral aetiology for rheumatoid arthritis (RA) has been much canvassed and investigated during the last 10 years but with negative results so far. Much of the search has concentrated on the fibroblast-like cells cultured from the RA synovial membrane or synovial fluid effusions and thought to be derived from type B or C lining cells (Smith and Hamerman, 1969). However, there is little evidence for viral genomes, productive viral infection, or neoantigens in such cells. For summaries and reviews see Hamerman (1975), Marmion and Mackay (1977), and Marmion (in press).

Less attention has been paid in RA to the possible production of disease as a result of the interaction of virus with cells of the immune and immunoregulatory system (Denman, 1975; Talal, 1977; Marmion and Mackay, 1977; Marmion, in press).

Reports on the relative proportion and classes of lymphocytes in rheumatoid synovial fluid and peripheral blood vary. Several investigations record an increase in T-cell numbers, a decrease in B-cell numbers (Froland *et al.*, 1973; Holborow *et al.*, 1975), and the presence of a population of null cells, up to 32% (Winchester *et al.*, 1974). Others have found an increase in B-cells (Mellbye *et al.*, 1972). The lymphocytes in RA synovial membranes, as distinct from the synovial fluid, are predominantly T-cells (Loewi, 1975).

There may be a marked diminution in responsiveness of synovial lymphocytes of RA patients to various mitogens, especially PHA (Stratton and Peter, 1972; Panayi, 1973; Holborow *et al.*, 1975) but reports are not consistent. Also the reactivity of peripheral blood lymphocytes in mixed lumphocyte culture may be depressed (Astorga and Williams, 1969). It is possible that the cells may already be activated or that the receptor sites for the antigen may be blocked; such effects might be virally induced.

In view of these pointers to disordered lymphocyte function in RA we decided to investigate RA synovial and peripheral blood lymphocytes for evidence of viral infection or of viral gene products. We were particularly interested in the association of retroviruses (type C or oncoviruses) with lymphocytes. This interaction has been well studied in various mice strains in which the immune response to endogenous viruses is modified by genetic factors involved with infectivity and expression (review, Ihle and Hanna, 1977). In human beings there have been extensive attempts to detect retroviruses, especially in association with leukaemia or lymphoma. Several isolations have been made, one of which was from cultured myeloid leukaemia cells. This virus was closely related to simian sarcoma virus and baboon endogenous virus (Reitz *et al.*, 1976), and it can be established as a chronic infection in various cell lines (Teich *et al.*, 1975).

A range of different cell-virus relationships exists with retroviruses and extends from productive infection to an integrated 'silent' genome. In addition some viruses are unable to replicate in cells of the same species from which they have been derived (xenotropic viruses). During the course of this study we used methods described by other workers for

Accepted for publication 19 December 1978.
Correspondence to Dr Mary Norval, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

successful culture of several xenotropic viruses. These involved fusing or cocultivating the lymphocytes with potentially permissive cells from another species. For example, baboon endogenous viruses have been isolated from normal baboon tissue by cocultivation with various permissive cell lines from rhesus monkey lung and dog thymus (Todaro *et al.*, 1974). In addition cultivation of integrated and silent retroviruses may require an activating step often involving turnover of cellular DNA or modification of DNA synthesis. One method of accomplishing this with lymphocytes is by a mixed lymphocyte reaction as demonstrated in mice (Hirsch *et al.*, 1972; Sherr *et al.*, 1974).

In the present investigation we first of all examined the synovial lymphocytes for any evidence of a productive infection with retroviruses by electron microscopy, scanning electron microscopy, labelling with ^{3}H-uridine, and culturing in conditioned medium, which has proved successful for sustained growth of human myelogenous leukaemia cells (Gallagher and Gallo, 1975). In addition methods were used to activate the lymphocytes before fusion and cocultivation with various cell lines which have proved permissive for primate retroviruses. The cells were subsequently examined for virus by labelling with ^{3}H-uridine, the culture supernatant being assayed for reverse transcriptase activity, and finally for the presence of virus-coded proteins in the cell membranes by immunofluorescence. In the accompanying paper the results of testing lymphocytes for the presence of viral antigens on their membranes, including retrovirus antigens by immunofluorescence and radioimmunoassay, are described (Hart *et al.*, 1979).

Materials and methods

SEPARATION OF LYMPHOCYTES

Specimens of paired synovial fluid and peripheral blood were obtained from patients attending the Northern General Hospital and were used within 2 hours of removal. Early in this study they were heparinised immediately (10 U/ml), but later the synovial fluid was left untreated while the peripheral blood was defibrinated with glass beads. Synovial fluids were treated with 80 U/ml hyaluronidase at 37°C for 30 minutes. Both were then separated on Triosil-Ficoll gradients (24 parts 9% Ficoll mixed with 10 parts 34% Triosil; centrifugation at 2000 r.p.m. for 20 min.). The lymphocyte-rich fraction was washed twice in Hanks's salt solution and counted after staining by the trypan-blue exclusion method or a special Boullard haematoxylin stain.

LYMPHOCYTE CULTURE

(*a*) The lymphocytes, generally at a concentration of around 10^6/ml, were routinely cultured in Earles-based Eagles complete medium (EE medium) containing 100 μg/ml streptomycin and 100 IU/ml penicillin supplemented with 20% autologous human serum. They were incubated at 37°C in 5% CO_2.

(*b*) ^{3}H-uridine labelling. The lymphocytes (from 2×10^6 up to 10^7) at a concentration of 10^6/ml were incubated overnight in EE medium containing 20% fetal calf serum or autologous serum or homologous plasma, plus (5-^{3}H) — uridine (20 μCi/ml) Amersham). Culture supernatants were concentrated using $(NH_4)_2SO_4$ (Grayzel, 1973), and were fractionated on sucrose gradients by the method outlined in Norval and Marmion (1976). In addition some lymphocytes were cultured for 4 days in the presence of phytohaemagglutinin (50 μg/ml) before the label was added.

(*c*) Cocultivation with fetal skin fibroblasts. Approximately equal numbers of lymphocytes were added to monolayers of human fetal skin fibroblasts cultured in EE medium plus 10% fetal calf serum. The medium was changed weekly and the cultures split fortnightly.

(*d*) Use of conditioned medium. The lymphocytes were cultured in conditioned medium from fetal skin fibroblasts; i.e., EE medium plus 10% fetal calf serum which had been in contact with the cells for about 4 days and which had been spun to deposit any cells.

(*e*) Colony formation in soft agar. 8×10^4/ml lymphocytes were mixed with complete 199 medium containing a final concentration of 0·3% agar and 20% human serum. In addition the lymphocytes were grown on prepared feeder layers. These comprised dextran separated mononuclear blood cells (10^6/ml) in complete 199 medium containing 0·5% agar and 20% human serum, and irradiated at 100 rad/min for 10 min (Goldman *et al.*, 1974; Prival *et al.*, 1974).

ELECTRON MICROSCOPY

Packed lymphocytes were prepared for electron microscopy after incubation overnight. The blocks were sectioned on a LBK Ultratome 11 and the sections viewed on a Hitachi HU11A electron microscope.

SCANNING ELECTRON MICROSCOPY

Lymphocytes after separation on Triosil-Ficoll gradients were washed in serum-free medium and prepared for scanning electron microscopy using the method of Alexander *et al.* (1976). The specimens were examined in a Cambridge stereoscan 180 microscope at 30 kV and 45° tilt.

POLYMERASE ACTIVITY

The synovial lymphocytes were assayed as described by Norval *et al.* (1975).

FUSION AND CO-CULTIVATION

(*a*) Stimulation of lymphocytes. Separated synovial fluid lymphocytes were stimulated in a mixed lymphocyte reaction (MLR). The preparation of lymphocyte pools, as stimulators of MLR, and the test have been described in detail by Urbaniak *et al.* (1978). Synovial fluid lymphocytes and stimulators were mixed in a 1:3 ratio. 0·5 μCi(^{3}H-methyl) thymidine (Amersham) was added to a test sample in a microtitre well after 3 days' incubation for 18 hours. Only those synovial fluid lymphocytes which showed positive MLR were used in the subsequent fusion. The remainder of the lymphocytes was stored in liquid nitrogen during the MLR test and used directly in the fusion.

(*b*) Polyethylene glycol (PEG) fusion and co-cultivation. Method as described by Pontecorvo (1975) with the modification that the PEG was drained off the cell sheet immediately. One batch of PEG (6000–7500 mol. wt., BDH), checked for toxicity to cultured cells, was used throughout. Three sets of lymphocytes were prepared: (i) 10^6 synovial fluid lymphocytes containing 3×10^6 stimulators, (ii) 10^6 synovial fluid lymphocytes, (iii) 3×10^6 stimulators.

(*c*) Sets from 7 patients with RA were used, and these were fused with 10^5 cells of the following cell lines known to support the growth of simian sarcoma virus and the baboon endogenous virus, which had been kindly provided by Dr N. Teich: A204 (human rhabdosarcoma), 7605L (human lung fibroblast), CCL64 (mink lung fibroblast) for 2 cultures; Tb-1-Lu (bat lung epithelial), CCL64 (mink lung fibroblast), D17 (dog fibrosarcoma) for 5 cultures. The medium used was EE plus 2, 5, or 10% fetal calf serum and culture was continued for a period of 8 weeks with the medium being changed twice weekly and the cells being split every fortnight. In one case the synovial fluid lymphocytes were pre-treated for 24 hours with 40 μg/ml IUDR plus 10^{-6}M diethylstilbestrol or IUDR plus 10^{-6}M hydrocortisone before fusion. After fusion culturing was continued in the presence of the appropriate hormone (Wu *et al.*, 1976). At various times the cultures were examined for evidence of retrovirus induction by:

(i) Assaying the reverse transcriptase activity of the culture supernatant at 3 and 7 weeks by the method of Dr N. Teich (personal communication).

(ii) Labelling with ^{3}H-uridine at 7 weeks followed by concentration of the culture supernatant and sucrose density gradient centrifugation as outlined by Norval and Marmion (1976).

(iii) XC test at 5 weeks (for 2 cultures only) by the method of Rowe *et al.* (1970).

(iv) Immunofluorescence at 7 weeks with various antiretrovirus antisera. Goat antisera to RD–114 (Tween-ether disrupted) and SSAV (Tween-ether disrupted) were originally obtained from Dr J. Gruber (National Cancer Institute), and used at 1/10 dilution in PBS, supplemented with 2% fetal calf serum (PBS–FCS). Rabbit antisera were later prepared to RD–114 and SSAV antigens (Hart *et al.*, 1979) and used at a dilution of 1/20 in indirect immunofluorescence tests on acetone-fixed cells grown on cover-slips (Schmidt *et al.*, 1966; Lennette *et al.*, 1967). Horse anti-human lymphocyte serum was kindly donated by Dr K. James. This serum was absorbed with acetone dried mink liver powder to give a working dilution of 1/4. Sheep anti-rabbit immunoglobulin and rabbit anti-horse immunoglobulin, both conjugated with fluorescein isothiocyanate (Wellcome Reagents), were used diluted to 1/8–1/16 in PBS–FCS. Rabbit anti-goat IgG FITC (Nordic Diagnostics Ltd.) was used at 1/8 dilution in PBS–FCS.

Results

ELECTRON MICROSCOPY

Five preparations of paired synovial fluid and peripheral blood lymphocyte from rheumatoid arthritis patients and 2 pairs from osteoarthrosis patients were examined after ultrathin sectioning. The lymphocytes from all sources looked normal, with large indented nuclei, compact chromatin, and a group of mitochondria opposite the indentation and a few mitochondria and ribosomes in the remaining cytoplasm. No membrane maturing or other viral-like structures were found. It was impossible to distinguish whether the lymphocytes were from the synovial fluid or the peripheral blood. Other cells seen included macrophages, monocytes, and a large number of platelets, particularly in the synovial fluids.

Four pairs of synovial fluid and peripheral blood lymphocytes from RA patients, 1 from juvenile RA, and 1 OA were examined by scanning electron microscopy. No surface features were found which distinguished cells from the 2 sources. Measurement of size showed that the lymphocytes from peripheral blood seemed to fall into 2 groups, which may correspond to T and B cells, while only the smaller size was present in the synovial fluid population. According to Polliack *et al.* (1973) the B cells in the blood are slightly larger than the T cells, so the smaller group common to both blood and synovial fluid may be T lymphocytes.

CULTURING OF LYMPHOCYTES

Initially synovial fluid lymphocytes and peripheral blood lymphocytes from 10 patients with RA, 1

with juvenile RA, and 5 non-RA were incubated overnight in medium containing 20% fetal calf serum or autologous serum or homologous plasma, and 20 μCi/ml ^{3}H-uridine in an attempt to label any RNA-containing virus which might be released into the fluid phase of the culture. Sucrose density gradient analysis of the concentrated fluid phase did not reveal labelled material at the appropriate density for a retrovirus. Rather more cell lysis occurred in the synovial fluid lymphocyte cultures than in the peripheral blood, particularly when calf serum or autologous human serum was used. This gave rise initially to patterns simulating virus release but the labelled material was sensitive to RNase and the release was less in homologous plasma.

Three cultures of synovial fluid lymphocytes from different patients were incubated first of all in the presence of phytohaemagglutinin for 4 days before adding ^{3}H-uridine. Again no peaks of labelled material typical of RNA viruses and retroviruses in particular were found.

As there did not appear to be a productive infection with an RNA virus in the synovial lymphocytes, the cells were examined for their DNA polymerase activity to determine if they contained any RNA-dependent DNA polymerase. Synovial fluid lymphocytes from 2 patients (1 RA and 1 possible juvenile RA) were assayed for polymerase activity and compared to a rheumatoid lymphoblastoid cell line kindly provided by Dr Ford (see below). The activity in all cases was found to be typical of that described in stimulated lymphocytes, that is an RNA-primed DNA-directed polymerase, not viral reverse transcriptase (Bobrow *et al.*, 1972). There was little stimulation by poly(rA).dT_{10}, and almost complete inhibition by actinomycin D.

It was noticed that if the cultures were kept for a longer period than overnight the lymphocytes gradually began to die, and after a month few viable cells remained. Human serum seemed to prolong the viability when compared to fetal calf serum, but this was only marginal. In order to examine the cells properly and to increase the numbers of synovial lymphocytes from any one patient, we tried various methods which have been successful in other systems. For example, conditioned medium from whole human embryo cells has been used to sustain growth of human myelogenous leukaemia cells in suspension culture (Gallagher and Gallo, 1975). Therefore the synovial fluid lymphocytes from 4 RA patients were cultured in conditioned medium prepared from fetal skin fibroblasts. The survival of viable cells was higher in this medium than in EE medium with 10% fetal calf serum over the first month, but subsequently they began to die and none survived longer than 2 months. Again, when synovial fluid lymphocytes from 4 RA patients were cocultivated with fetal skin fibroblasts, they survived as viable cells throughout 2 months but were not dividing. The culture supernatant was tested at 4 and 8 weeks for reverse transcriptase activity and was negative in all cases.

Ford and Smiley (1973) reported the spontaneous occurrence of a continuous line of lymphoblastoid cells which appeared during culture of an RA synovial membrane. These cells contain EBV genome and synthesise IgG of the specificity of rheumatoid factor in culture. However, attempts to transform synovial fluid lymphocytes from 4 RA patients with EBV by the method of Miller *et al.* (1974), known to be successful with cord blood lymphocytes, were failures.

GROWTH OF LYMPHOCYTES IN SOFT AGAR

Lymphocytes from 3 RA synovial fluid specimens stored in liquid nitrogen and 3 normal peripheral blood lymphocytes were cultured in soft agar with and without a feeder layer of peripheral blood mononuclear cells inactivated with *x*-rays. All plates were viewed after 10 days. A few cells proliferated in plates without feeder layers (Table 1), but by day 12 these were no longer detected. Substantially more clusters were seen on the plates with feeder layers but never any colonies. ('Clusters' were considered to contain 10–20 cells and 'colonies' more than 50). Also there were no differences in the numbers of clusters formed between normal peripheral blood and rheumatoid synovial fluid lymphocytes. Clusters from one synovial fluid lymphocyte culture (number 2 in Table 1) were fixed for electron microscopic examination. The cell type could not be identified from the cellular debris, but no viral particles were seen in this dividing population of cells.

Table 1 *Numbers of 'clusters' of cells seen on day 10 after culturing lymphocytes in soft agar with and without a feeder layer of peripheral blood mononuclear cells irradiated at 100 rad/min for 10 min*

	Number of 'clusters' on day 10 (2 *plates*)			
	Without feeder layer		*With feeder layer*	
Normal peripheral blood lymphocytes				
1	0	0	8	7
2	0	0	7	4
3	0	0	7	12
Rheumatoid synovial fluid lymphocytes				
1	1	2	8	12
2	3	4	9	10
3	0	0	3	1

PEG FUSION

As no proliferation or long-term culture of synovial lymphocytes was obtained using the above methods, it was decided to try activating the lymphocytes and then to fuse them with cell lines which had proved permissive for a variety of primate retroviruses. Only those cells able to be activated by a MLR were used for the subsequent fusion and cocultivation.

In the first place the efficiency of fusion when PEG was used was determined by forming heterokaryons of synovial lymphocytes and CCL64 mink cells. Two days after PEG treatment the cells were stained by immunofluorescence by means of anti-human lymphocytic serum.

Heterokaryons were frequent and were mainly binuclear, with numerous long appendages which were not detectable in mononuclear cells (Fig. 1). The heterokaryons were not present at the end of the 7-week culture.

After activation the synovial fluid lymphocytes were fused with 3 out of a selection of 5 cell lines, A204, 7605L, CCL64, Tb-1-Lu, and D-17, and the cultures carried for an 8-week period with periodic testing for evidence of retrovirus expression. In 1 instance the lymphocytes were pretreated with IUDR and either diethylstilbestrol or hydrocortisone before fusion. In all, synovial fluid lymphocytes from 7 patients were used. These patients had RA for periods ranging from 1 year to 27 years, and varied from seronegative to seropositive.

The cultures were followed using several methods. Firstly, the supernatant was assayed for reverse transcriptase activity, and in all cases this was negative, indicating that the cells were not producing retroviruses. A positive control of culture supernatant from RD cells infected with RD–114 virus was always included in the samples to be assayed. In addition labelling with ^{3}H-uridine followed by sucrose density gradient centrifugation did not show any peak at the density of a retrovirus, i.e., 1·16–1·18 g/cm^{3}, again indicating that there was no productive virus infection. In some cases an XC test was carried out. This has been used to detect murine leukaemia virus genome in cells as these will form plaques when placed in contact with XC cells (rat tumour cells transformed by Rous sarcoma virus) (Rowe *et al.*, 1970). Plaques and syncytia were never formed when the fused cells were cultured in the presence of XC cells. The method was checked by using mouse 3T3 cells infected with AKR type of murine leukaemia virus.

The final test involved immunofluorescence on acetone-fixed cells grown on coverslips which should detect retrovirus membrane antigens, if these are being expressed in the fused cells. Antisera to the viruses RD–114 and SSAV were chosen. The first is an endogenous cat virus with a close antigenic relationship to the baboon endogenous virus, and the second is a simian sarcoma virus. Both share various antigenic determinants, such as p30 and gp69–71, with other mammalian species and should therefore be able to detect any retroviral antigens in human cells. Originally goat antisera to the 2 viruses, which had been Tween-ether disrupted, were used, but later antisera prepared in rabbits were used (Hart *et al.*, 1979). In no case was there any suggestion of positive immunofluorescence.

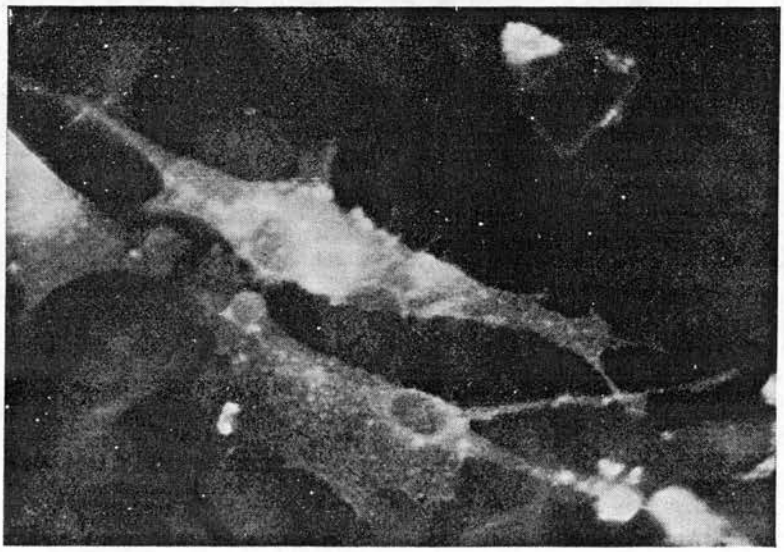

Fig. 1 *Heterokaryons formed by PEG fusion of synovial lymphocytes with CCL 64 cells, stained with horse anti-human lymphocyte serum and anti-horse FITC conjugate to demonstrate a human nucleus within a mink lung fibroblast and human antigens on the cell surface.*

Discussion

In the past few years much attention in RA research has focused on the type B synovial lining cell and its presumed derivative in culture, the synovial fibroblast. Although these cells have some abnormal characteristics when compared to fibroblasts from other disease conditions such as osteoarthrosis, there is no evidence of neoantigens on the cell surface, either virally induced or arising from mutation.

In this paper we have attempted to look at the lymphocytes present in the synovial fluid effusions of patients with RA to see if they contain RNA viral genes that might be linked with the chronic inflammatory state of the joint.

It seemed unlikely, especially in the light of the thorough attempts by various groups of workers to isolate retrovirus from human leukaemia cells, that the synovial lymphocytes would be productively infected with a virus, and, indeed, our morphological results after ultrathin sectioning did not indicate any abnormalities in these cells compared to those from the peripheral blood, and there was no evidence of virion production. Similarly the surface of the lymphocytes did not differ from the 2 sources when examined by scanning electron microscopy. Labelling with ^{3}H-uridine also did not indicate a productive viral infection.

We turned then to more indirect methods of detecting viral information in RA lymphocytes. It is of interest in this context that a retrovirus, SP104, has been associated with canine systemic lupus erythematosus (SLE), and by immunofluorescence viral membrane antigens have been demonstrated on some peripheral blood lymphocytes in human SLE (Schwartz, 1975). In addition, an antigen, p30, related to mammalian type C viral protein has been localised in renal glomeruli in human SLE (Mellors and Mellors, 1976). More recently, however, viral mRNA of SP104 was not detected in either human or canine SLE tissues by sensitive methods of nucleic acid hybridisation (Quimby *et al.*, 1978), a result which is hard to reconcile with the previous data. In the accompanying paper Hart *et al.* (1979) describe experiments to detect retrovirus antigens on the membranes of synovial lymphocytes by radioimmunoassay and immunofluorescence with uniformly negative results. However, possibly it was necessary to culture the cells to induce the virus, and indeed, as in the case of xenotropic viruses, which will replicate only in cells of a different species, it might have been necessary to use methods of induction, fusion with permissive cells, and cocultivation before virus is isolated.

Our attempts to culture the synovial lymphocytes were not successful, using different species of serum, conditioned medium, cocultivation with fibroblasts, or colony formation in soft agar. The latter would have indicated transformation of a lymphocytic population. Finally, then, the lymphocytes were stimulated by an MLR and fused using PEG with a variety of cell lines known to be permissive for several primate retroviruses. Heterokaryons were formed which were monitored over 8 weeks by several methods, ^{3}H-uridine incorporation, reverse transcriptase activity of the culture supernatant, XC test, and immunofluorescence with antisera to RD–114 and SSAV, which should detect mammalian interspecies and group antigens. All results were negative.

Thus in this systemic study we have not been able to detect any viral information in RA synovial lymphocytes and very few differences in their characteristics compared to peripheral blood lymphocytes of the same patient.

We wish to thank Mrs Alexia Graham, who did the transmission and scanning electron microscopy reported in this paper, and Miss Sheena Tuach for expert technical assistance. The help of Mrs Leonora Hislop and the staff at the Rheumatic Diseases Unit, Northern General Hospital, in providing clinical specimens is gratefully acknowledged. Grant support throughout this work was given by the Nuffield Foundation.

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Annals of the Rheumatic Diseases, 1980, **39**, 159–163

Search for viruses in rheumatoid macrophage-rich synovial cell populations

HELENA HART AND MARY NORVAL

From the Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG

SUMMARY The adherent cells remaining after short-term culture of synovial fluid and synovial membrane cells from rheumatoid and non-rheumatoid patients were examined for the presence of a productive virus infection and for various viral antigens. Labelling was carried out with ^{3}H-thymidine and ^{3}H-uridine followed by sucrose density gradient centrifugation of the culture supernatant. Only in 1 case was there incorporation of ^{3}H-uridine into material of density 1·21 g/cm^3. Viral antigens were tested for by indirect immunofluorescence with antisera to rubella virus, the retroviruses RD–114 and simian sarcoma associated virus, early adenovirus type 2 antigens, late adenovirus type 2 antigens, SV–40 T antigen, and in 1 case measles virus. No cell showed immunofluorescence with any antiserum except the early adenovirus type 2 antiserum, which stained the cytoplasm of about half the synovial cell cultures, some from rheumatoid and some from non-rheumatoid patients.

A possible viral aetiology of rheumatoid arthritis has been suggested (Denman, 1975; Barland, 1973; Hamerman, 1975; Marmion and Mackay, 1977), but it has not yet been supported by clear experimental evidence. Virological investigations until recently have concentrated on the long-term culturing of fibroblastic cells. These are derived from synovial membranes of fluids and are thought to be either type A or B lining cells (Smith, 1971). These studies included attempted detection of cytopathic and non-cytopathic viruses, viral genomes, and neoantigens, and no evidence was found which implicated any virus (Person *et al.*, 1973; Grayzel, 1973; Norval and Marmion, 1976).

An alternative hypothesis (Talal, 1975; Denman *et al.*, 1976; Marmion and Mackay, 1977; Fundenberg and Wells, 1976) suggests that the primary and central change is in the effector cells of the immune system (T or B lymphocytes or monocyte-derived macrophages) and that the rheumatoid process involves a loss of the normal immunoregulatory control rather than a normal immune reaction against an altered target cell such as a virus-infected fibroblast. This loss of immunoregulatory control could be due to a viral infection of the immune effector cells (Messner, 1974).

Recent virological studies on lymphocytes from synovial fluids and membranes have included an examination for retroviruses, but these were not shown to be present by methods such as nucleic acid hybridisation, fusion, and cocultivation with cells permissive for primate retroviruses, immunofluorescence, and radioimmunoassay (Norval *et al.*, 1979; Hart *et al.*, 1979).

The other type of effector cells of the immune system remaining to be subjected to experimental scrutiny is the monocyte derived macrophage. These are the adherent cells present within the first few days of culture of synovial fluids as described by Mackay *et al.*, (1974) which do not seem to multiply and cannot be subcultured. Most often they are overgrown after variable periods of time by the fibroblastic cells. In addition disaggregated synovial membranes yield a heterogeneous mixture of fibroblasts, macrophages, and undifferentiated esterase-positive cells on primary cultures, and again the fibroblasts generally overgrow the other cells. Traycoff *et al.* (1976) have described methods for identifying the various populations of synovial mononuclear cells on the basis of morphology, sudanophilia, and several cell markers, and they classified such cells into synovial lining cells, monocyte-derived macrophages, monocytes, lymphocytes, and lymphoblasts.

The role of the synovial macrophages in the joint is not clear. It has been suggested that they may be bearing foreign antigens, as antibody dependent lymphocyte cytotoxicity to adherent cells has been

Accepted for publication March 16, 1979
Correspondence to Dr M. Norval

demonstrated in short-term cultures (Neill, personal communication). It is of interest that macrophage-lymphocyte clusters have been described in autologous cell mixtures obtained from the synovial fluid of rheumatoid patients (Hepburn *et al.*, 1974). Webb *et al.* (1975) showed that such clusters could be formed with heterologous lymphocytes also. This suggested that there may be an antigen on the macrophages towards which the lymphocytes are sensitive, although there was no stimulation of the lymphocytes in these clusters. It is possible that such an antigen may be virally induced or coded.

Thus it was thought worthwhile to examine the synovial macrophages for the presence of a productive viral infection by means of short-term cultures and labelling with ^{3}H-uridine and ^{3}H-thymidine. In addition these cells were examined for various viral antigens by immunofluorescence.

Materials and methods

CELL CULTURE

Synovial fluids from patients with classical or definite rheumatoid arthritis (RA), other forms of inflammatory joint disease, and osteoarthrosis (OA) were treated within 2 hours of extraction from the patient with 80 U/ml hyaluronidase for 30 min at 37°C. The cells were deposited by centrifugation at 800 *g* for 10 min and washed once in Earles-based Eagle's complete medium (EE). Short-term cultures were set up using 1–2 $\times$ 10^7 viable cells/ml EE; 10 ml into 100 ml flasks for labelling with ^{3}H-thymidine and ^{3}H-uridine, and 1 ml into each tube containing coverslips (22 $\times$ 6 mm) for immunofluorescence. Flying coverslips were used to estimate the number of adherent cells, and 1 ml cell suspension was sufficient to prepare 8 coverslips. All cultures were incubated for 3 hours at 37°C in air containing 5% CO_2. The non-adherent cells were removed at this stage by washing thoroughly twice in EE.

Synovial membranes obtained at synovectomy were disaggregated within 2 hours of the operation as described by Abrahamsen *et al.* (1975), and the single cell suspensions were then handled as above.

COUNT OF ACID ESTERASE-POSITIVE ADHERENT CELLS

The flying coverslips were fixed in Baker's formol calcium and the acid esterase staining procedure was carried out as described by Mueller *et al.* (1975). The adherent count (AC) was determined by counting the acid esterase-positive cells (AEPC) on 10 mm^2 of the coverslip and calculating as follows:

AC/ml cell suspension = 10 $\times$ 13·2 $\times$ 8 $\times$ AEPC count.

VIRUS DETECTION

After washing the cell sheet to remove the non-adherent cells, the flasks were labelled by adding 10 ml EE containing 5% fetal calf serum and either (5-^{3}H)-uridine or (methyl-^{3}H)-thymidine (Amersham), both at a final concentration of 10 μCi/ml. Incubation was continued for a further 20 hours at 37°C. On 3 occasions attempts were made to induce a virus by adding cyclohexidimide (10μg/ml) at same time as the radioactive medium.

Detection of virus in the culture supernatant was performed as outlined by Norval and Marmion (1976).

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This was carried out as described previously (Hart and Marmion, 1977) with coverslips fixed in acetone after the 3 hour incubation. The specific antisera used were all prepared in rabbits and have been described before together with appropriate control anticell sera. They were anti-rubella virus (Thomas strain) grown in RK_{13} cells (Hart and Marmion, 1977); anti-RD114 grown in RK_{13}, and anti-simian sarcoma virus grown in KNRK cells (Hart *et al.*, 1979); anti-adenovirus type 2 grown in RK_{13} cells treated with cytosine arabinoside (10 μg/ml) and used early in infection, and anti-adenovirus type 2 grown in RK_{13} cells used late in infection (Jones *et al.*, 1979).

In one experiment guinea-pig anti-measles serum (Flow Laboratories) was used. A weak but definitely positive reaction was detected specifically on measles infected Vero cells at 1/20 dilution. This serum was used at 1/10 dilution for the synovial cells. A few immunofluorescent tests also included hamster anti-SV 40 T antigen serum kindly donated by Dr E. Rogers.

Fluorescein-conjugated anti-rabbit Ig (Wellcome Laboratories) was used at 1/16 dilution: fluorescein-conjugated anti-guinea-pig and anti-hamster (Nordic Diagnostic Laboratories) were used at 1/8 dilution.

Results

CULTURE TECHNIQUES

Cells from synovial fluids or membranes of 14 patients with rheumatoid arthritis (RA), 4 with osteoarthrosis (OA), 5 with other non-RA conditions, and 1 undiagnosed were used and the adherent cell population separated after culturing for 3 hours at 37°C. The clinical details are listed in Table 1. In cases where there were sufficient cells, the cultures were labelled with ^{3}H-thymidine and ^{3}H-uridine, and, after concentration by precipitation with $(NH_4)_2SO_4$, the culture supernatants were analysed

by sucrose density gradient centrifugation. The acid-insoluble counts per minute in each fraction were determined and plotted against the density of the sucrose to find out if there was a peak of incorporation at any particular density throughout the gradient. As may be seen from Table 1, only in one case, 780127, was there a peak of radioactive insoluble material after labelling with ^{3}H-uridine, which occurred at density 1·21 g/cm^3. This is shown in Fig. 1. Labelling of the same culture with ^{3}H-thymidine did not produce such a peak, supporting the idea that there might have been production of an RNA-containing virus by the adherent synovial cells from this patient. The density, 1·21 g/cm^3, is similar to that found on ^{3}H-uridine labelling of measles virus produced from Vero cells (Norval and Smith, 1979).

With some synovial specimens there were not sufficient cells to enable the labelling experiment to be carried out, and only immunofluorescence was done.

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Coverslips containing adherent cells from all the specimens listed in Table 1 were fixed and stained with specific viral antisera to rubella virus, RD–114 (a feline endogenous retrovirus), SSAV (a simian sarcoma retrovirus), and adenovirus type 2. With the first 3 antisera no specific immunofluorescence was seen, though occasionally there was a non-specific staining, but this could be easily recognised as the control anti-cell sera reacted similarly. In addition results with the antiserum to the late adenovirus type 2 antigens were uniformly negative. However, with the antiserum to the early adenovirus type 2 antigens about half the specimens examined showed weak uniform non-granular cytoplasmic immunofluorescence, which was not present when the control anti-cell serum was used. This immunofluorescence had no particular pattern being present in the adherent cells from some synovial membranes and some fluids, and from approximately equal numbers of RA patients and non-RA.

Table 1 *Clinical and laboratory details of patients whose synovial specimens were used as a source of adherent cells. Results indicating the count of acid esterase positive adherent cells/ml in the population, and labelling of culture supernatants with ^{3}H-thymidine and ^{3}H-uridine followed by sucrose density gradient centrifugation are shown. In addition immunofluorescence using a variety of viral antisera was carried out on all the specimens*

Case number	*Diagnosis*	*Tests for rheumatoid factor*		*Specimen*	*Count of acid esterase positive adherent cells/ml*	*Labelling of culture supernatants with*	
		SSCT	*Latex*			*^{3}H-thymidine*	*^{3}H-uridine*
780103	RA	128	..	SM	..	..	..
780104	RA	1024	+++	SF	..	—	—
780105	RA	32	..	SF	..	..	..
780106	OA	..	..	SF	..	..	..
780107	RA	256	+++	SF	..	—	—
780108	Psoriatic arthropathy	<16	—	SF	..	—	—
780109	RA	..	..	SM	..	—	—
780110	RA	<16	—	SM	..	—	—
780114	RA	64	++	SF	..	—	—
780115	OA	..	..	SM	$3{\cdot}2\times10^5$	—	—
780117	RA	16	..	SM	..	—	—
780118	RA	<16	±	SF	$1{\cdot}1\times10^5$	—	—
780119	Undiagnosed	..	..	SF	$7{\cdot}5\times10^3$	—	—
780121	Bilateral congenital dislocated hip	..	..	SM	$1{\cdot}4\times10^5$	..	..
780122	OA	<16	..	SM	$4{\cdot}8\times10^4$	—	—
780123	RA	<16	++	SM	$1{\cdot}8\times10^4$	..	..
780124	Synovitis	<16	—	SF	$1{\cdot}5\times10^4$	..	..
780127	RA	32	..	SM	..	—	Peak at density 1·21 g/cm^3
780129	OA	..	..	SM	..	—	—
780130	RA	<16	—	SF	..	..	..
780131	Psoriatic arthropathy	<16	—	SF	$9{\cdot}1\times10^4$	—*	—*
780132	RA	1024	..	SF	$6{\cdot}7\times10^4$	..	—*
780133	RA	<16	..	SF	$4{\cdot}2\times10^4$	—*	—*

* 780131 and 780133 were also treated with 10 μg/ml cycloheximide at the same time as adding ^{3}H-thymidine or ^{3}H-uridine; 780132 was treated with cycloheximide at the same time as adding ^{3}H-uridine.

.. = not tested. SM = synovial membrane. SF = synovial fluid. RA = rheumatoid arthritis. OA = osteoarthrosis. SSCT = (sensitised sheep cell test)—agglutination of sheep red cells coated with specific rabbit antibody. Latex = agglutination of latex particles coated with IgG globulin.

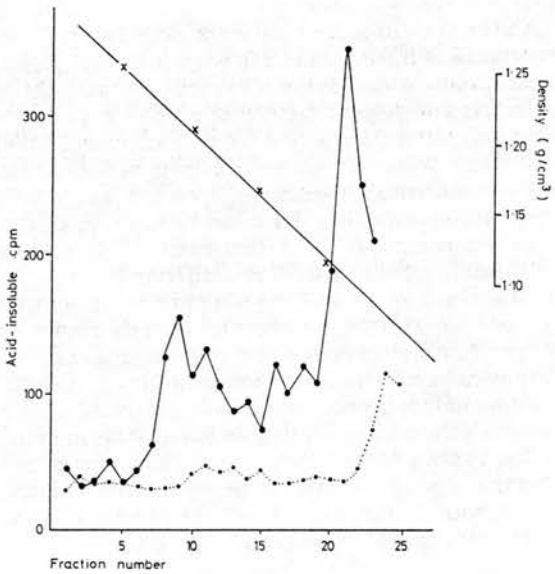

Fig. 1 *Acid-insoluble counts per minute in sucrose density gradient fractions from 780127 adherent cell culture supernatant after labelling with ^{3}H-thymidine (●----------●) and ^{3}H-uridine (●———●).*

Three cultures, 780131, 780132, and 780133, were also reacted with anti-SV 40 T antigen serum but with negative results.

Finally, measles antiserum was used on adherent cells from patient 780127 to check whether the peak obtained on sucrose density gradients after labelling with ^{3}H-uridine represented productive measles infection. No measles antigens were detected, and no further identification of the material within the radioactive peak was made.

Discussion

Very little has been published thus far on the properties of the synovial monocyte-derived macropages, though there have been suggestions that they may express unique antigens or phagocytose specific immune complexes and thus may account for the immune processes seen in RA.

Rheumatoid synovial macrophages and lymphocytes were shown to form rosettes or clusters in both autologous and heterologous systems, but, as the lymphocytes were not stimulated by this process, there was no indication from this work that an antigen was present on the macrophage surface towards which the lymphocytes were sensitised (Hepburn *et al.*, 1974). This result was confirmed by Runge (1976), who measured lymphocyte stimulation after culturing synovial macrophages with autologous peripheral blood lymphocytes from RA patients and controls. A small degree of stimulation was observed in the rheumatoid cultures, but this was not significantly different from that observed in non-RA cultures. It was suggested that there may be non-specific lymphocyte activation by immune complexes associated with the macrophages rather than a specific synovial antigen expressed on the macrophages.

On the other hand the work of Neill (personal communication) does support the idea that some rheumatoid synovial cells at very early passes may express unique antigens. These were detected by an antibody-dependent cytotoxicity technique using synovial cells of mixed types as targets and autologous serum. On subculture, which would eliminate the macrophage population, the cytotoxicity became negative. Other work using similar techniques and also giving negative results has involved target synovial cells maintained in culture through several subcultures, so that no macrophages were likely to be present (Griffiths *et al.*, 1976).

In this present study an attempt was made to look for virus production and some viral antigens on the adherent synovial cells remaining after a short-term culture. It was hoped in this way to examine a macrophage-rich population of synovial cells which are eliminated by the usual methods of in-vitro culture. A productive virus infection was not demonstrated apart from one instance in which there was incorporation of ^{3}H-uridine into material of density 1·21 g/cm^3, the same as measles virus. However, by immunofluorescence using anti-measles serum no measles antigens were expressed on the adherent cells from this patient. The culture was not analysed further.

Further, when the adherent cells were examined by immunofluorescence for rubella virus antigens, retrovirus antigen, SV–40 T antigen, and adenovirus type 2 late antigens, they were all negative. Thus a unique antigen, induced or coded for by these viruses, was not expressed on the synovial macrophage population. Of course many other virus groups remain, but the ones tested here represent good candidates, being implicated in persistent infections and tumorigenicity.

There was some immunofluorescent staining with antiserum to early adenovirus type 2 antigens in the cytoplasm of the adherent synovial cells from some patients. This did not seem to correlate with the diagnosis of the patient or the type of specimen. It may represent expression of some adenovirus genes in normal human tissue which has been described recently in placentas by Jones *et al.* (1979), and which is under investigation.

We thank the surgeons and clinicians at the Princess Margaret Rose Hospital and the Northern General Hospital Hospital and Mrs Leonora Hislop for providing the synovial specimens used in this study.

Grant support was given by the Nuffield Foundation.

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Cover-slip cell cultures

Sir,

We were interested in the Short Communication by Marshall, Lamey & Ferguson[1] which described an autoradiography technique applicable to cover-slip cell cultures. For some time now we have been doing *in situ* hybridizations using radioactively labelled viral nucleic acids as probes on cover-slip cell cultures, followed by autoradiography. We use a method described below which seems to eliminate several of the steps outlined by Marshall *et al.*

After the hybridization the cover-slips are washed thoroughly in buffer at 4°C and then dehydrated through alcohols. They are mounted on DPX on microscope slides with the

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cells uppermost, and allowed to dry at room temperature overnight before being dipped in photographic emulsion. After exposure and developing, the cover-slips are stained and dehydrated as normal. They can be viewed directly microscopically, or protected for long-term storage by placing another cover-slip on the top and sealing around the edge with DPX. By this method the cover-slips are handled only at the initial stage and several can be placed on the same slide, thus saving emulsion.

Mary Norval and W. A. Neill

Department of Bacteriology,
University Medical School,
Teviot Place,
Edinburgh, EH8 9AG, Scotland.

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IM INTERNAL MEDICINE FOR THE SPECIALIST — Rheumatology

A Viral Etiology for Rheumatoid Arthritis?

By Mary Norval, Ph.D.

Substantive evidence of a viral etiology for rheumatoid arthritis remains elusive, but the possibility continues to intrigue scientists and clinicians. Features of viral infections that might induce chronic arthritis are difficult to prove by standard techniques, but immunological methods may provide an indirect means of seeking a viral etiology. Dr. Norval presents virologic and immunological considerations in the continuing search for a viral etiology and discusses specific viruses implicated in rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic disease of joints that currently affects 1 to 2 percent of the adult population. Studies on very early RA cases are rare because of difficulties in making an accurate diagnosis and in obtaining appropriate specimens from patients, but histopathological observations indicate some proliferation of synovial-lining cells, initially with lymphocyte infiltration around synovial vessels. Later, immunoproliferation of the synovial membrane occurs with greatly increased local synthesis of immunoglobulins. This immune pathogenesis has suggested the idea of local antigenic stimulation as being crucial in the etiology of the disease. Over the past two decades, great efforts have been made by sci-

Dr. Norval is a lecturer, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, United Kingdom.

entists and clinicians alike to discover such a specific antigen or agent, but, as yet, no totally convincing evidence has been uncovered.

Theories of an infective etiology for RA have ranged from the view that the initial event is infection of the joint by an agent leading to either some kind of persistent infection or some altered immune response, or both, to the view that the initial event is a change in immunoregulatory control that may lead to an altered response to an infecting agent.[1] In addition, it is now recognized that there is a genetic component in RA, with an increased incidence of some HLA antigens, specifically HLA-D4 and HLA-DR4, in RA patients and in healthy persons with a strong family history of RA.[2] This has led to speculation about these antigens and the ability of various agents, particularly viruses, to interact with genetically determined features of the cell surface. Alternatively, some evidence exists to support the opinion that infecting agents are not involved at all in the initiation or progression of RA and that the whole disease is caused by altered immune mechanisms.

In this review, current evidence is summarized that implicates viruses as the etiologic agents in RA; a brief overview is given of virologic and immunological aspects, followed by a section on general methods of study in this difficult area; and finally, specific virus groups are detailed in which there is interest at present.

GENERAL VIROLOGIC CONSIDERATIONS

An ideal agent in RA must be ubiquitous, arthrotropic, and capable of altering immune responses, e.g., causing the production of rheumatoid factors, which are found in a substantial proportion of patients. Some viruses have properties that would make them good candidates. It is known, for example, that infections with such viruses as Epstein-Barr virus (EBV), coxsackie virus B, and rubella virus can induce acute polyarthritis, which persists for a long time and during which infectious virus also persists. The polyarthritis, however, does not generally progress to chronic destructive joint disease. If viral infections are considered as the initial event in RA, several scenarios are possible. First, there could be an initial infection that is followed by elimination of the virus but also a persisting altered immune response. This would make a search for the agent very difficult in established cases of RA. Second, chronicity of the virus, either locally in the affected joints or elsewhere in the body, could lead to altered immune mechanisms, such as altered "homing" properties of subpopulations of lymphocytes. Some viruses have been shown to persist for life after the primary infection, either by a low-grade infection of a few cells that is probably controlled by the immune response (e.g., EBV) or by an integration of their genomes into host-cell chromosomes, thus becoming latent (e.g., herpes simplex virus). Third, a primary change in the effector cells (T- and B-lymphocytes or macrophages) of the immune system could lead to a loss of normal immunoregulatory control, which might also lead to an altered response to a virus, especially a virus capable of persisting in the body. Each of these hypotheses has some evidence to support it at the present time.

GENERAL IMMUNOLOGICAL CONSIDERATIONS

One of the distinguishing features of RA is the large number of cases that are seropositive for rheumatoid factors. Most commonly, these rheumatoid factors are autoantibodies against the Fc portion of immunoglobulin G (IgG). It is not known whether they arise from abnormal handling of a particular virus that may trigger the disease by locally stimulating antiglobulin production, or whether there is already an immunoregulatory defect that leads to production of rheumatoid factors perhaps an abnormal response to a virus at the same time.

At the end of 1983, an interesting hypothesis was proposed by Plotz[3] that explains a role for viruses in the pathogenesis of autoimmune diseases. Plotz speculated that antiidiotypic antibodies (i.e., antibodies directed against novel sequences in the variable portion of immunoglobulins), directed against antibodies with specificity for viral components that bind to cell surfaces, may also act as antibodies against cell-surface components. Thus, autoantibodies in various diseases may actually be antiidiotypic antibodies to antiviral antibodies and have additional properties of acting as antibodies against various host-cell structures. The autoantibodies so far characterized are all directed against structures that viruses may require for nucleic-acid replication or protein synthesis. It is tempting to speculate that some autoantibodies in RA might be just such antiidiotypic antibodies to an infect-

ing virus. It will be of great interest to see what evidence can be gathered in support of this hypothesis.

Recently, it has been found that seronegative RA patients have serum antibodies directed against rheumatoid-factor idiotypes.[4] It is possible that such antibodies could lead to the elimination or inactivation of the precursor B cells for rheumatoid factors. Certainly, the blood of seronegative patients is deficient in precursor B cells that can be stimulated by a polyclonal activator (e.g., EBV), lending support to this idea. It is not known as yet whether these serum antibodies are present in seropositive RA patients.

If viral infections are considered as the initial event in RA, several scenarios are possible. First, there could be an initial infection that is followed by elimination of the virus but also a persisting altered immune response. Second, chronicity of the virus, either locally in the affected joints or elsewhere in the body, could lead to altered immune mechanisms. Third, a primary change in the effector cells (T- and B-lymphocytes or macrophages) of the immune system could lead to a loss of normal immunoregulatory control.

It is known that some autoantibodies are often generated after viral infection. The most recent evidence concerning these has come from a study of reovirus infection in mice.[5] Surprisingly, the autoantibodies produced after the infection had specificity for both infecting virus and host, implying shared antigenic structures between viral determinants and normal tissues, and suggesting a possible mechanism of host-cell damage. There may be a number of self-reactive B-cell clones that are normally regulated to prevent development of autoimmunity. The virus may alter this balance in some way, perhaps by its cytotoxic effect. These concepts could be applied to the pathogenesis of RA, and further characterization of the generation of autoimmunity after viral infection may lead to fresh ideas on tissue damage in the rheumatoid joint.

METHODS OF STUDYING VIRUSES IN RA

A combination of direct and indirect approaches has been used to try to elucidate a viral etiology for RA. The most obvious method, perhaps, is to follow what happens during acute polyarthritis caused by known viruses, although it is recognized that such a condition rarely progresses to chronic erosive arthritis. Studies with rubella virus and EBV are of particular concern in this context.

By direct examination of diseased synovial tissue in the electron microscope, virus particles have been seen on three occasions, and were always present in very small numbers with differing morphology. None of these reports have been substantiated. Direct attempts have also been made to isolate viruses from rheumatoid joints, either from synovial membrane or from synovial-fluid specimens. This approach can be taken further by co-cultivating or fusing synovial cells directly from the patient with tissue-culture cells that are known to be permissive for a particular virus. Isolations are very rare, and, even if viruses are found, it would not be possible to determine whether they are initiating agents or merely secondary invaders, perhaps resulting from the abnormal inflammatory state of the joint. Synovial cells or extracts of them have also been introduced into experimental animals. Notably, in 1984, this led to the isolation of parvoviruses, after injection of rheumatoid synovial material into the brains of suckling mice.

A few studies have included detection of viral antigens on synovial cells by immunofluorescence, immunoperoxidase, or cytotoxicity tests. Even fewer studies include detection of viral nucleic acids by hybridization. These approaches are of particular interest, because it is now recognized that some viruses can associate with cells in a nonproductive manner, without causing their death. Regrettably, such work has been predominantly negative. If cells are already infected by a virus, they may be resistant to superinfection by it or a related virus. This method has also been tried with rheumatoid material, especially

peripheral-blood lymphocytes, without notable success. In addition, the presence of neoantigens on synovial cells has not been demonstrated in cytotoxicity tests using autologous peripheral-blood lymphocytes, and neither is it suggested by the histopathology of early RA.

Various immunological methods, which have been used to provide indirect evidence for viral involvement in other diseases, have been tried in RA. Serum antibody levels to a range of common viruses are not generally different between RA patients and control groups, although some recent work with cytomegalovirus (CMV) antibodies in early RA is of interest. In addition, finding higher antibody levels to various EBV antigens in RA patients has provided the basis for arguments advocating a role for EBV in RA. Local synthesis of viral antibodies in the synovium has not been found, and viral antibodies have not been eluted from synovial tissue, where they might reasonably expect to be bound if antigens are present. By measuring stimulation of synovial lymphocytes by various viruses, no consistent proliferative response has been obtained, indicating that there is probably no population present already sensitized to the viruses used. Experiments have been carried out on *in vitro* synthesis of immunoglobulins by synovial-membrane cells and on lymphocyte function in RA by measuring, for example, delayed hypersensitivity or nonspecific mitogen responses. No clear results were obtained with either method. It is now possible to analyze the lymphocyte subsets in the rheumatoid synovium, by using such methods as monoclonal antibodies that are specific for subpopulations and fluorescence-activated cell sorter, and to compare these with the peripheral blood. Such work should yield informative data in the near future. Also, various groups are currently examining the antiidiotypic hypothesis, by testing RA sera for their ability to block or adsorb specific antiviral-antibody activity, for example, or to block infection of normally susceptible cells by specific viruses.

Animal models have proved generally disappointing in RA research, although, in goats, there is chronic progressive arthritis with demyelinating encephalomyelitis associated with a retrovirus that is of considerable interest.[7] Transmission experiments have most often failed, including one reported in 1983, in which rheumatoid synovial cells were injected into

Various immunological methods that have been used to provide indirect evidence for viral involvement in other diseases have been tried in RA

Several surveys have indicated, however, that antibodies to rubella were no more prevalent in RA patients than in controls. Eluates from RA synovial membranes did not contain rubella antibodies. There are few successful isolations of rubella from RA synovial-fluid specimens, and no rubella antigens or viral nucleic acid are detectable in RA synovial-membrane tissue. It is therefore possible that rubella may persist in the joints in a latent form and that it is reactivated at intervals by some inflammatory stimulus. Unfortunately, no good evidence exists, as yet, to suggest that it is associated with progression to a chronic, erosive joint disease with rheumatoid factor production.

Cytomegalovirus. CMV, a member of the herpesvirus group, persists for life after the primary infection occurs in unknown site(s) in the body, although it can be isolated on occasion from peripheral-blood leukocytes. CMV reactivates at intervals and, because of these properties plus the disturbance of T-lymphocyte subsets during CMV mononucleosis, is worth considering in the context of RA.

There has been only one report of isolation of CMV from the synovial cells of an RA patient who had no clinical signs of CMV.[10] It was impossible to tell if CMV merely happened to be present in this site, perhaps in some inflammatory cell, as has been shown in the colonic mucosa. Circulating antibodies to early CMV antigens have been demonstrated in 20 percent of early RA patients but have rarely been found in more established cases.[11] This may indicate some defect in handling CMV, but it seems more likely that these results can be attributed to the general immunoproliferative process of RA.

Retroviruses. There has been much curiosity recently about this group, particularly after the 1980 discovery of the human T-cell leukemia viruses (HTLV) and their possible association with acquired immunodeficiency syndrome.[12] HTLV can use HLA antigens as receptors to enter host cells and can also avoid the cytotoxic immune response by this mechanism. Further retroviruses are able to persist in their hosts in an integrated genomic form with little or no expression of viral proteins. These properties make them potential etiologic agents in RA, especially considering the association between HLA antigens and the arthritic diseases.

Disappointingly, although there has been one report of seeing viral particles with typical morphology of this group in rheumatoid synovial membranes, no viral antigens, viral nucleic acid, or reverse-transcriptase activity has been found in rheumatoid synovial tissue. It would be interesting to repeat these studies with reagents prepared from HTLV instead of the primate retroviruses used until now.

As already mentioned, a retrovirus, caprine arthritis-encephalitis virus, belonging to the lentivirus subfamily, has been isolated from goats with chronic progressive arthritis.[7] Work is in progress using the caprine, viral nucleic acid and antigens as probes to determine if a lentivirus is present in RA patients.

EBV. EBV is another herpesvirus and is the causative agent of infectious mononucleosis. It is ubiquitous and persists for life after the primary infection, probably in a subpopulation of B-lymphocytes. EBV stimulates production of various immunoglobulins, including rheumatoid factors and has been suggested as an autoimmune trigger in RA.[13] A study of antibody levels to various EBV antigens in RA patients and controls indicates that there may be a more active infection in RA or perhaps an altered immune response to the virus. In 1976, an antibody was detected in sera from 90 percent of RA patients directed toward an antigen induced in lymphocytes by EBV infectin.[14] This was called RA-associated nuclear anti-

gen (RANA) and was found in 15 to 56 percent of sera from normal persons and at a lower titer than in the RA patients. Further work to look for this antigen in synovial cells from RA joints was disappointing: it was rarely found, although the antibody was commonly present. EBV antigens and viral nucleic acid were also not detected in affected joints.[15] Recently, RANA and the nuclear antigen induced by EBV in infected cells (EBNA) have been shown to be one and the same protein; apparent differences in antibody titer in RA sera toward RANA and EBNA perhaps resulted from differences in avidities of the antibodies.[16]

Nevertheless, although localized EBV infection of the synovium in RA cannot be demonstrated, there are differences in lymphocyte responsiveness between RA and control populations.[17] When lymphocytes from EBV-infected individuals were incubated with EBV, there was an initial increase in immunoglobulin-secreting cells, followed by suppression by T cells. When lymphocytes from RA patients were used, no suppression occurred. Thus, in RA, there may be a defect or absence of T cells that regulates the infected B cells, or the impaired response of rheumatoid B cells to normal, regulatory T cells. EBV may then be important in chronicity and progression of the joint disease through increased antibody production, thereby perpetuating the inflammatory process. Analysis of T-cell subsets in RA synovial fluid and membranes is currently being undertaken[18] and should yield significant results, especially if specimens can be obtained from early stages of the disease (before treatment starts).

New theories of autoantibody and antiidiotypic-antibody production following viral infection are of particular interest in the context of RA

CONCLUSIONS

No definitive evidence has been produced so far to show that any virus is the etiologic agent in RA. Various findings over the years have temporarily raised hopes, but these have not been substantiated. Nevertheless, several viruses are seen as potential candidates, and more information is becoming available about their properties and interactions with humans using modern techniques of molecular virology and immunology. New theories of autoantibody and antiidiotypic-antibody production following viral infection are of particular interest in the context of RA. Perhaps it is too naive to think of a single factor, such as one individual virus, causing RA, and a combination of circumstances may be crucial (e.g., the patient's genetic background and immune status at the time of the infection). Some definite answer will surely be forthcoming within the next few years. IM

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Archives of Virology 67, 203—215 (1981)

Archives of Virology

Association of Human Cytomegalovirus (HCMV) with Mink and Rabbit Lung Cells

By

Helena Hart and Mary Norval

Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh, Scotland

With 6 Figures

Accepted October 15, 1980

Summary

The association of human cytomegalovirus with mink and rabbit lung cells was studied. Strain AD-169 was used which was free of Mycoplasma and other contaminating agents. It was found to be incapable of productively infecting mink lung cells. Infection appeared to be initiated but aborted at an early stage. This was indicated by indirect immunofluorescence, assays of culture supernatants and cell lysates for infectious virus, electron microscopy of ultra-thin sections of infected cells, labelling of virus and viral DNA with ^{3}H-thymidine and assay of virally-induced DNA polymerase at various times after infection. On the other hand, using these methods, AD-169 was found to infect rabbit lung cells, the virus being produced in low amounts over a period of up to one month after infection. At this time, focal areas of infection were still apparent and 15 per cent of the cells expressed nuclear viral antigens as shown by immunofluorescence. The viral genome was assumed to have become latent in some rabbit cells with a few being capable of producing infectious virus.

Introduction

Human cytomegalovirus (HCMV) normally replicates in the nuclei of human fibroblasts, although there have been reports of other cells being susceptible to infection. Two human systems have been described; firstly, lung epithelial cells being productively infected with slow release of virus over several weeks and accompanying cytopathic effect typical of cytomegalovirus (12); and secondly, amnion epithelial cells being productively infected, the virus yield being lower than from equivalent fibroblasts and the replication cycle being slower (4). In addition, human embryonic kidney epithelial cells which are normally non-permissive for cytomegalovirus replication can be converted into a permissive state by treatment with iododeoxyuridine before infection (15).

0304-8608/81/0067/0203/$ 02.60

Various non-human systems have also been described in attempts to find a permissive cell for HCMV replication. Guinea-pig embryo cells were abortively infected, with no new virus particles being produced (5). Early virus antigens were formed and cellular DNA synthesis stimulated. However, the DNA polymerase activity in the infected cells did not have the properties of the enzyme induced by HCMV in permissive cells (8). Mouse fibroblasts can also be abortively infected, the block in replication being at the level of viral DNA synthesis (1).

In addition, DARAI and FLUGEL (2) found that epithelial-like mink lung cells were highly susceptible for the replication of varicella-zoster and suggested that this might be a suitable cell line for the growth of other Herpes viruses, like Epstein-Barr virus and cytomegalovirus.

Finally FARBER *et al.* (3) discussed the replication of HCMV in rabbit lung fibroblasts. They noted a cytopathic effect in this system after 5 to 7 days which increased for 2 weeks and then remained unchanged over a period of up to 28 days. CMV antigens could be detected in the nucleus in 40 per cent of the cells by immunofluorescence at this time. Although no infectious virus was detected in the culture supernatant 3 days after infection, virus could be recovered by co-cultivation of the rabbit lung cells with human embryo lung cells even 28 days post-infection.

We decided to investigate further the association of HCMV with rabbit lung cells and to ascertain whether mink lung cells could be infected. This included an examination of infected cells by electron microscopy, growth curves over periods of up to 20 days, labelling with ^{3}H-thymidine and subsequent extraction of viral DNA, immunofluorescence over periods of 32 days and finally measurement of DNA polymerase activity inside infected nuclei.

Materials and Methods

Cells

Rabbit lung cells were prepared by trypsinization of the lungs of a 1 year old New Zealand White rabbit and grown in Earles based Eagles medium (MEM) containing 100 i.u./ml penicillin and 100 µg/ml streptomycin and supplemented with 2 to 5 per cent rabbit serum. After the cells had formed monolayers they could be split 1:3 every week and were fibroblastic in appearance. They were used between pass 8 and 20.

Mink lung cells (Mv-1-Lu) were obtained from the American Type Culture Collection, number CCL 64, and were cultured in MEM containing 2 to 5 per cent new born calf serum and antibiotics as above. They were epithelial-like in appearance and extensive microvilli on the cell surface could be seen on electron microscopy of ultra-thin sections.

Human embryo lung cells (HEL) were prepared as described for the rabbit lung cells except that the medium was supplemented with 5 to 10 per cent new born calf serum instead of rabbit serum.

Incubation of all cells was at 37° C in an atmosphere of air containing 5 per cent CO_2. All cells were free of contamination with Mycoplasma.

Virus

Human cytomegalovirus (HCMV), strain AD-169, which had been plaque purified by Dr. J. M. DeMarchi and was free of other contaminating viruses as tested by Dr. G. Darai, was obtained from Dr. J. Cameron, Institute of Virology, Glasgow. It was also free of contamination with Mycoplasma species.

Infection of Cells

Cells were infected as monolayers, allowing 1 hour for adsorption at 37° C, or in suspension with gentle shaking for 2 hours at room temperature. The cells were washed before maintenance medium containing 2 per cent of the appropriate serum was added.

Ultrastructure

Cells were harvested using glass beads and prepared for electron microscopy. The blocks were sectioned on an LKB Ultratome 11 and the sections viewed on a Hitachi HU 11 A electron microscope.

Immunofluorescence

An indirect immunofluorescence test was performed on acetone-fixed (10 minutes at 4° C) cells grown on cover-slips using a variety of human convalescent sera which had a high complement fixing titre to cytomegalovirus. Sheep anti-human immunoglobulin conjugated with fluorescein isothiocyanate (Wellcome Reagents) was added, diluted 1/10 in phosphate buffered saline (0.01 M pH 7.2) containing 2 per cent foetal calf serum to reduce non-specific staining.

Growth Curve of AD-169 in Rabbit Lung, Mink Lung and HEL Cells

For this experiment, the rabbit lung cells were first adapted to grow in the presence of new born calf serum instead of rabbit serum, as the former was found to be toxic for HEL cells on subsequent assay.

2×10^6 cells were put into 5 mm Petri dishes (Sterilin) in 5 ml medium and incubated overnight. They were infected at a multiplicity of 0.3, and the incubation continued over a period of 20 days with a change in maintenance medium every 4 days. At intervals, plates were removed, the culture supernatant was collected and the trypsinised cells frozen and thawed twice followed by ultrasonication for 1 minutes to release cell-associated virus. Both were then assayed according to the method of HAMELIN and LUSSIER (7) using HEL cells in Microtitre plates (Nunc).

Labelling of Virus with ^3H-Thymidine

At various times after infection of cells with AD-169, the medium was replaced with MEM containing 1 per cent serum and 2.5 μCi/ml (methyl-^{3}H) thymidine (The Radiochemical Centre, Amersham), and incubation continued for a further 48 hours. The culture supernatant was harvested, clarified at $10{,}000 \times g$ for 10 minutes and then either precipitated with an equal volume of cold saturated $(NH_4)_2SO_4$ or centrifuged at 30,000 rpm for 1 hour in an SW 40 rotor The deposit was suspended in a small volume of buffer, 0.01 M Tris-HCl, 1 mM-EDTA, pH 7.1 (6). It was layered on to sucrose density gradients (20 to 70 per cent) and centrifuged at 25,000 rpm for 18 hours in an SW 40 rotor. Fractions were collected from the base of the tube, precipitated with cold 10 per cent TCA and counted after filtration through glass fibre discs (14). Under these conditions HCMV has a density of 1.219 g/ml (10).

Labelling of Viral DNA with ^3H-Thymidine

Cells were grown in 4 roller bottles (2 51) until almost confluent (about 2×10^7 cells per bottle), then 2 infected with AD-169 at a multiplicity of 1, and 2 left as controls. 5 days post-infection, (methyl-^{3}H) thymidine was added as described above. 48 hours later, the culture supernatant was collected and cells removed with glass beads. After clarification of the culture supernatant the virus particles were deposited by centrifugation at 30,000 rpm in an SW 40 rotor. They were resuspended in a small volume of buffer and purified on a sucrose gradient as above. The cells were frozen and thawed twice followed by ultrasonication and the cell-associated virus particles similarly purified on sucrose density gradients. The fractions from the gradients with density 1.20 to 1.23 g/ml were pooled, diluted and the virus particles deposited by centrifugation at 40,000 rpm for 1 hour in an SW 50 rotor. The pellet was treated with pronase, 2 mg/ml (Boehringer), and SDS, final concentration 1 per cent, at 37° C for 8 hours and the viral DNA banded on a 10 to 40 per cent sucrose density gradient (10). Fractions were collected from the base of the tube, precipitated with cold 10 per cent TCA and counted after filtration through glass fibre discs.

DNA Polymerase Assays

The infected cell lysates were prepared and the assay carried out according to the method described by HIRAI, FURUKAWA and PLOTKIN (8), except that activated calf thymus DNA was used in place of salmon sperm DNA as a template, and each assay contained 10 μCi (methyl-^{3}H) thymidine 5′-triphosphate (30 Ci/m mole; The Radiochemical Centre, Amersham). The nuclear fractions (5—30 μl) were used to assay the

Fig. 1. Thin section of AD-169 infected rabbit lung cell showing an enlarged and involuted nucleus (×4800)

DNA polymerase activity. At 15 minutes intervals during incubation, 50 µl samples were removed, spotted on to Whatman filter paper discs which were then fixed in cold 10 per cent TCA washed five times in cold 10 per cent TCA then twice in cold ethanol, before being air-dried and counted in PPO-toluene scintillator in a Packard Tri-Carb liquid scintillation instrument. Protein content of the samples was estimated by the method of LOWRY *et al.* (11).

To distinguish viral DNA polymerase from cellular DNA polymerase, $(NH_4)_2SO_4$ at a final concentration of 54 mM was added to the assay mix (8).

Results

Cellular Changes After Infection

When AD-169 was used to infect rabbit and mink lung cells at a multiplicity of infection of 0.1 to 3, there was an effect on the cells easily seen by light microscopy and appearing first 2 to 3 days post-infection. The rabbit cells became rounded and piled up in certain areas. This effect persisted for up to 2 months. The mink cells also became rounded although, in this case, there were no focal areas of infection. The cells continued to divide and the effect gradually disappeared over a 3 week period.

Ultra-thin sections of both cell types were examined in the electron microscope at 5 and 15 days post-infection. No abnormalities or viral structures could be seen in the mink lung cells at these times. However, in the rabbit lung cells, although no whole virus particles or dense bodies could be seen, the nucleus often looked abberrant, being enlarged and involuted. This is illustrated in Fig. 1.

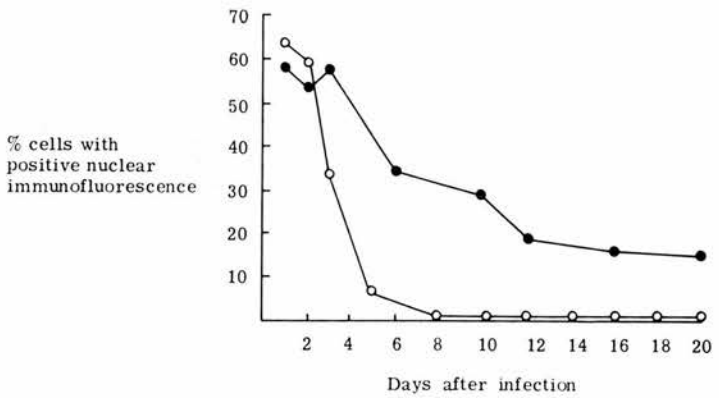

Fig. 2. Percentage of rabbit lung (•———•) and mink lung (○———○) cells showing positive nuclear immunofluorescence at various times after infection with AD-169

Immunofluorescence

Indirect immunofluorescence was carried out on AD-169 infected rabbit and mink lung cells at various times up to 20 days post-infection, using 3 selected human sera which gave nuclear immunofluorescence with AD-169 infected human embryo lung cells. The infection was at a multiplicity of 1. In the mink lung cells, there was positive immunofluorescence showing initially as uniform nuclear stain-

ing of over half of the cells, but this number decreased rapidly and all were negative by 6 days post-infection. In contrast, in the rabbit lung cells, positive immunofluorescence was seen in 50 per cent of the cells 1 day after infection and, although this number diminished, 15 per cent were still positive 20 days after infection. These results are shown in Fig. 2. The cells were not subcultured during this time. Initially, the positive immunofluorescence was associated with in-

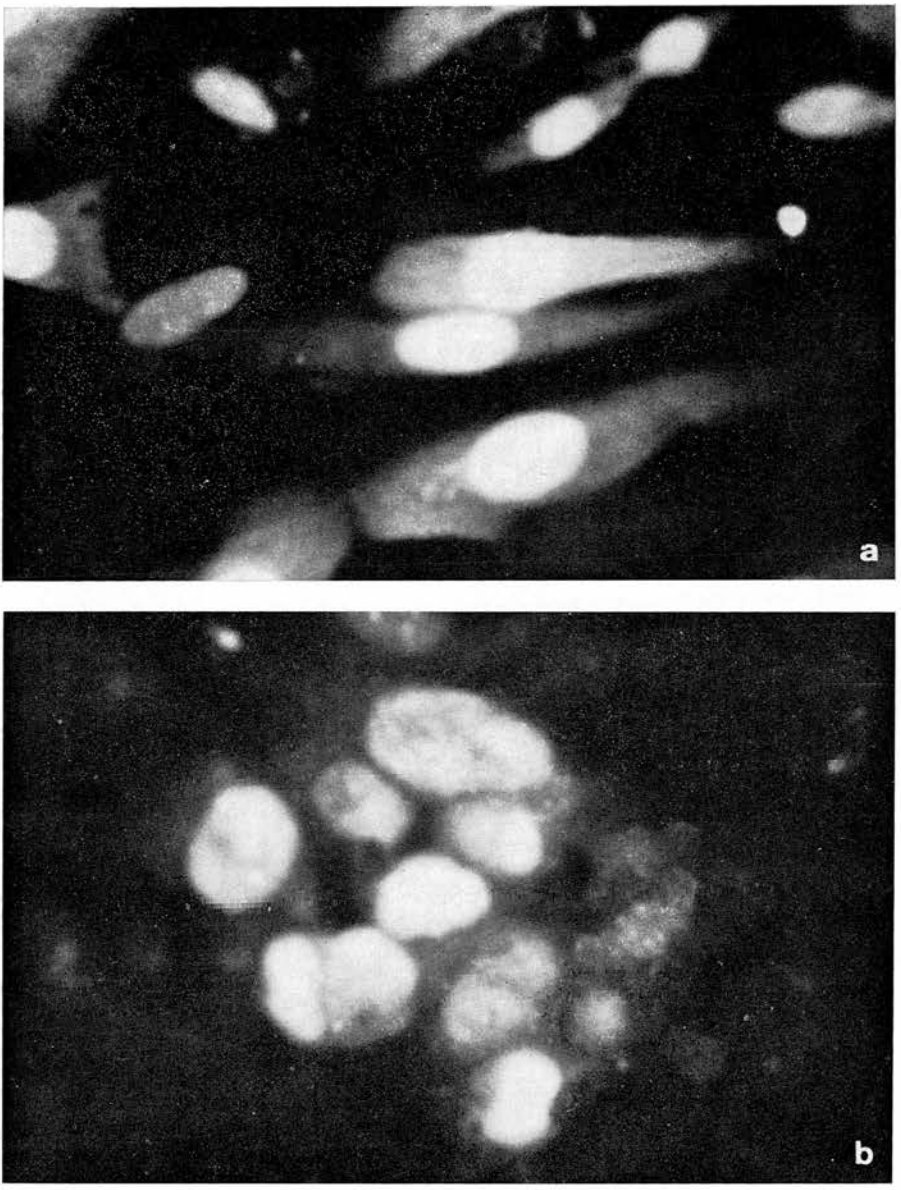

Fig. 3. Indirect immunofluorescence on AD-169 infected rabbit lung cells *a* 2 days post-infection and *b* 10 days post infection

dividual cells (Fig. 3a) but several days post-infection the positive cells were in a form of multinuclear giant cells (Fig. 3b).

In addition to indirect immunofluorescence, anti-complement immunofluorescence was carried out on the infected rabbit lung cells and gave the same picture, the cells being cultured for 32 days in this case.

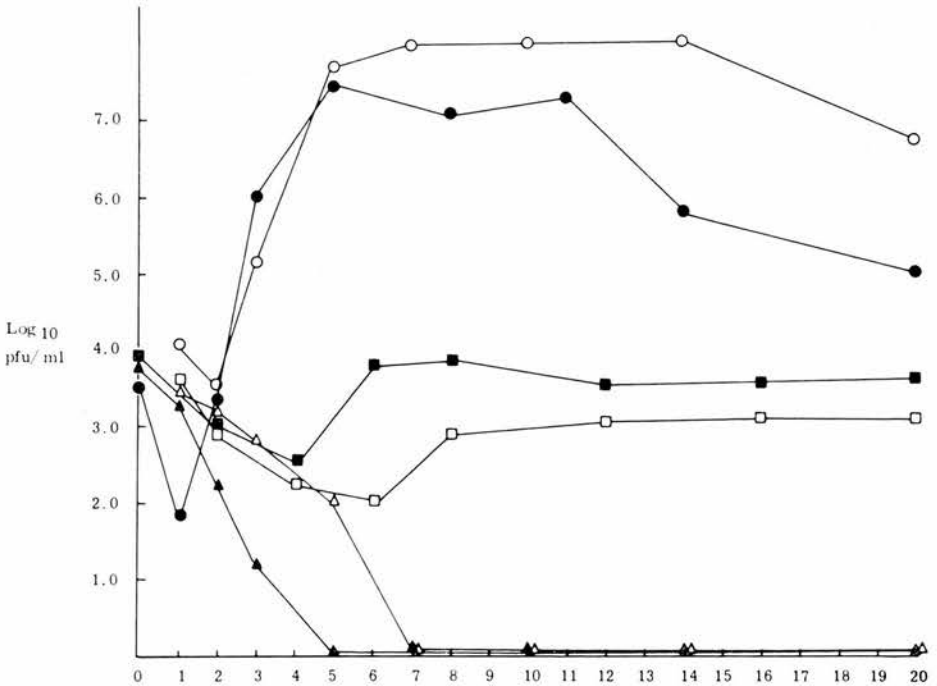

Fig. 4. Assay of infectious AD-169 in culture supernatant and cell-associated at various times after infection. (Human embryo lung cells; supernatant ●——●, cell-associated ○——○; mink lung cells, supernatant ▲——▲, cell-associated △——△; rabbit lung cells, supernatant ■——■, cell associated □——□)

Growth Curves

Culture supernatants and cell lysates of mink and rabbit lung cells were assayed at various times post-infection with AD-169 with the result shown in Fig. 4. Infected HEL cells were used as a positive control. It may be seen that, in the case of the mink lung cells, the titres of virus in the culture supernatant and cell-associated dropped rapidly until no infectious virus was apparent 6 days after infection. The growth curve of AD-169 in rabbit lung cells showed a different picture. There was a fairly steady and low level of infectious virus both in the culture supernatant and cell-associated which persisted as such over the 20 day period of the experiment.

Labelling of Virus and Viral DNA with ^{3}H-Thymidine

^{3}H-thymidine was added to infected rabbit, mink and human embryo lung cells between 5 and 7 days post-infection, with subsequent assay of the culture

supernatant by sucrose density gradient centrifugation for labelled acid-insoluble material of the density of CMV, i. e. 1.22 g/ml. While a large peak of radioactivity was obtained at this density in the case of the culture supernatant from infected HEL cells, no labelling was found in the culture medium of infected mink lung cells. With the rabbit lung cells, again there was no peak of labelling at this density. However, the picture in this case was not entirely clear due, it is thought, to the substantial degree of cell lysis which occurred in these cells when infected. This resulted in some labelling at a slightly lighter density than CMV and which may be sufficient to mask a small amount of viral-labelled material.

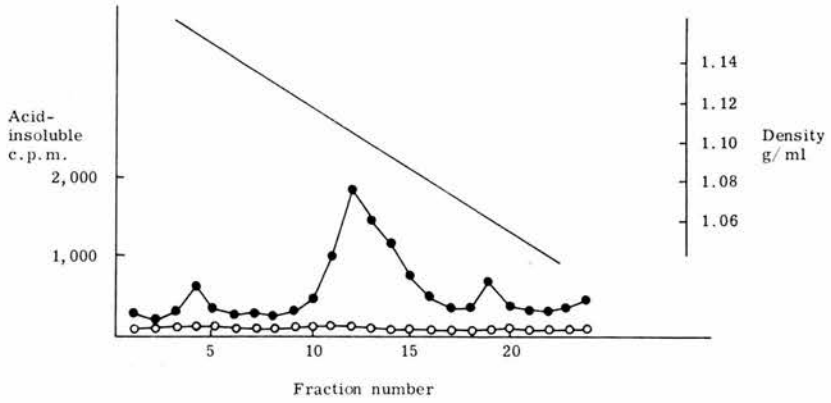

Fig. 5a

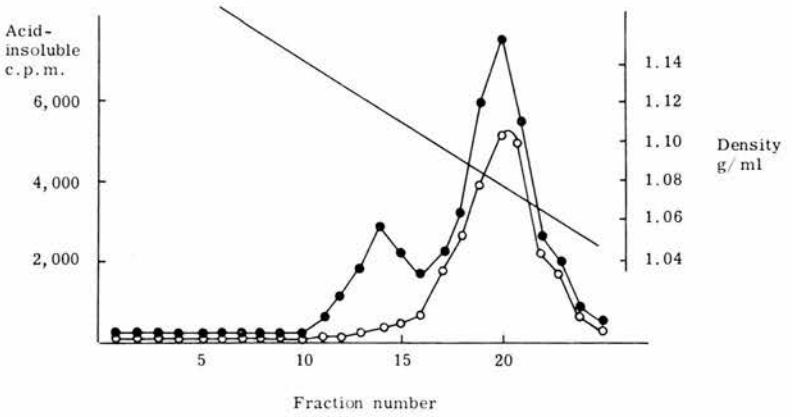

Fig. 5b

To clarify this situation, the material with density 1.20 to 1.23 g/ml was taken from these gradients and the viral nucleic acid, if any, extracted and subsequently banded on a 10 to 40 per cent sucrose gradient. In addition to the culture supernatant, cell lysates were also examined in this way. The result may be seen in Fig. 5.

Using HEL cells infected with AD-169, a peak of labelled acid-insoluble material with density around 1.10 g/ml was found both in the culture supernatant

(Fig. 5a) and cell-associated (Fig. 5b), which was not present in uninfected control cells. The same experiment using mink lung cells showed no such peak, indicating that viral DNA was not being replicated in these cells (Figure not shown). With the rabbit lung cells, no labelling was apparent at this density in the cell lysates (Fig. 5d) but a small peak was seen in the material from the culture supernatant (Fig. 5c). This may represent small amounts of DNA in virions released from infected cells.

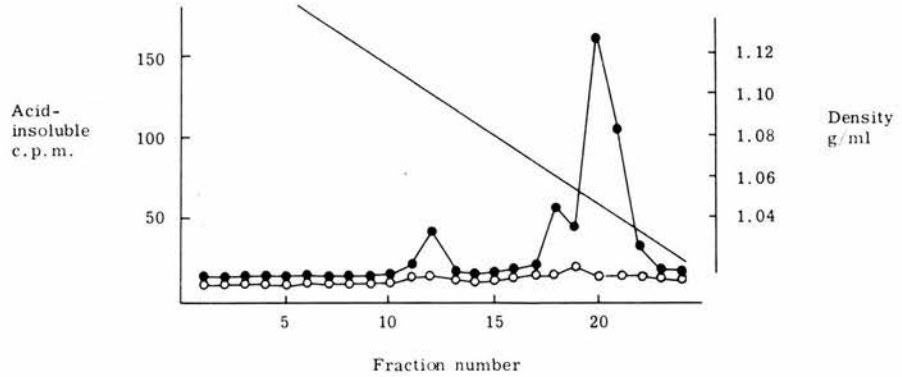

Fig. 5c

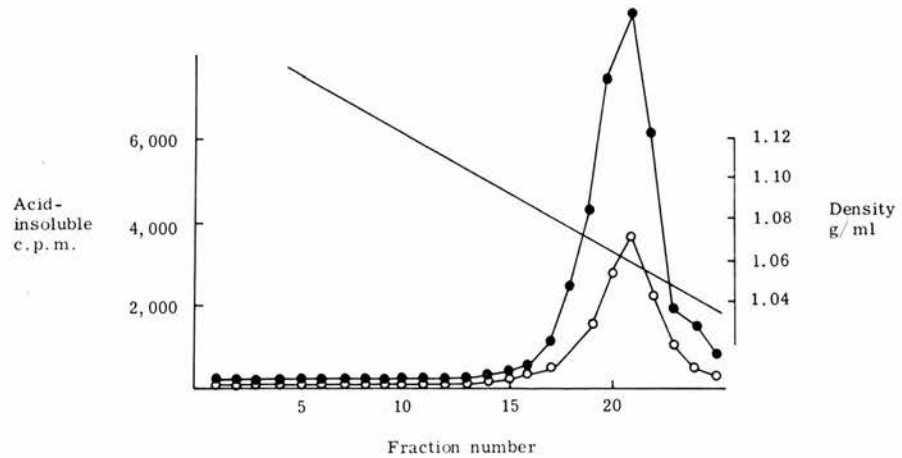

Fig. 5d

Fig. 5. Cells infected with AD-169 and uninfected controls were labelled with ^{3}H-thymidine, the virus was purified from the culture supernatants and cell lysates, its DNA extracted and run on a sucrose density gradient. The graphs show the acid-insoluble counts per minute (cpm) of the fractions from the gradient *a* culture supernatants of human embryo lung cells (○——○) and human embryo lung cells infected with AD-169 (●——●), *b* lysates of human embryo lung cells (○——○) and human embryo lung cells infected with AD-169 (●——●), *c* culture supernatants of rabbit lung cells (○——○) and rabbit lung cells infected with AD-169 (●——●), and *d* lysates of rabbit lung cells (○——○) and rabbit lung cells infected with AD-169 (●——●)

DNA Polymerase Activity

It has been reported that DNA polymerase induced by HCMV is different from the normal cellular DNA polymerase (8, 9). In particular, it was found to be stimulated by concentrations of 50 to 100 mM $(NH_4)_2SO_4$, which inhibit cellular DNA polymerase activity.

The infected cells were harvested at various times after infection and the nuclear fractions prepared. These were assayed with and without 54 mM $(NH_4)_2SO_4$ and the counts per minute (cpm) of ^{3}H-TTP incorporated into acid-soluble material in 45 minutes per μg protein was calculated. A typical assay curve for HEL and mink lung cells is shown in Fig. 6 (a) and (b) and the total results are given in Table 1.

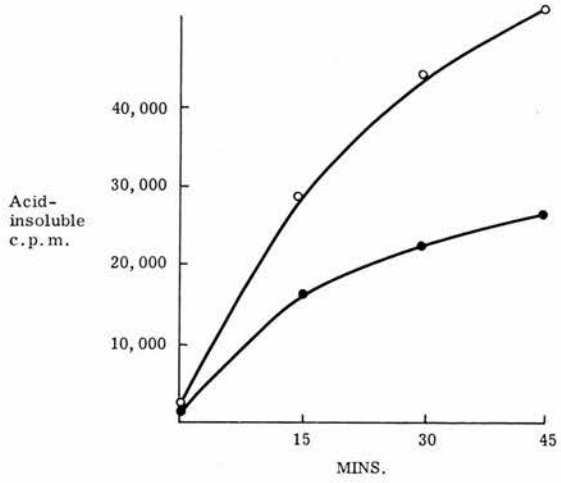

Fig. 6 a

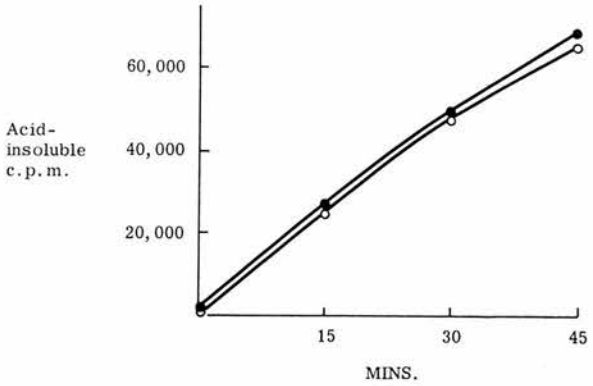

Fig. 6 b

Fig. 6. *a* DNA polymerase activity of nuclear fraction (90 μg protein) from AD-169 infected human embryo lung cells with (○———○) and without (●———●) 54 mM $(NH_4)_2SO_4$. *b* DNA polymerase activity of nuclear fraction (200 μg protein) from AD-169 infected mink lung cells with (○———○) and without (●———●) 54 mM $(NH_4)_2SO_4$

Considering HEL-infected cells, there was an increase in DNA polymerase activity 4 days post-infection by about 15-fold compared to the uninfected cells. This enzymatic activity was stimulated over 2-fold by the presence of 54 mM $(NH_4)_2SO_4$ in the assay mix while the control activity was inhibited. In infected mink lung cells DNA polymerase activity was stimulated, a peak of 12-fold 2 days after infection and gradually decreasing thereafter. However, this enzyme seemed to be inhibited by $(NH_4)_2SO_4$ at all times. A very similar picture emerged for the rabbit lung cells with a 2-fold increase of DNA polymerase activity 5 days post-infection, and there was no stimulation by $(NH_4)_2SO_4$.

Table 1. *DNA polymerase activity of nuclear fractions of human embryo lung (HEL), mink lung and rabbit lung cells, and after infection with AD-169*

	Increase in acid-insoluble cpm in 45 mins/μl	Protein μg/ml	Increase in acid-insoluble cpm in 45 mins/μg protein	Increase in acid-insoluble cpm in presence of $(NH_4)_2SO_4$ in 45 mins/μl	% activity with $(NH_4)_2SO_4$ / activity without $(NH_4)_2SO_4$
HEL cells	460	7.8	59	336	73
4 days after infection	16,200	17.8	910	33,690	208
Mink lung cells	120	2.9	41	108	90
1 day after infection	1,750	6.3	278	1,950	111
2 days after infection	3,350	6.6	508	3,240	97
5 days after infection	3,000	8.9	337	1,500	50
10 days after infection	600	5.0	120	600	100
Rabbit lung cells	250	2.6	96	175	70
2 days after infection	533	3.8	140	500	93
5 days after infection	1,000	4.8	208	1,225	109
10 days after infection	290	2.2	132	272	94

Discussion

We were interested to understand the association of AD-169 with mink and rabbit lung cells. All experiments detailed here indicated that mink lung cells were not capable of supporting replication or persistence of HCMV. There was an initial toxic effect, followed by recovery of the cells within a few days. Immunofluorescence results indicated that infection was initiated but aborted at an early stage as no positive cells remained 6 days after infection. Growth curves of virus in the medium and the cells corroberated this. No virus or viral-induced structures were seen on electron microscopy of ultra-thin sections and there was no induction of viral DNA polymerase activity detectable in infected cells. In addition, no labelling of virus or viral DNA with ^{3}H-thymidine in infected cells was obtained.

It has been reported (3) that rabbit lung cells may be infected with AD-169, the virus being produced for the first 3 days after infection, and not thereafter, although it could be recovered by co-cultivation with human embryo cells even after 28 days in culture. Immunofluorescence showed 40 per cent of the cells with nuclear fluorescence at this time and in 10 per cent it was thought that some early

proteins had accumulated to give large fluorescent granules, perhaps indicating a block in replication. Our experiments would support the idea of a long-term persistent infection of rabbit lung cells with HCMV, but in our system small amounts of infectious virus were released from the cells at a fairly constant rate over 4 weeks after infection. This could be measured by assay of the culture supernatant for infectious virus, and also by assay of viral DNA in the culture supernatant after labelling with ^{3}H-thymidine. Measurement of DNA polymerase activity did not indicate induction of an enzyme with the properties of HCMV DNA polymerase. However, this is probably not a sufficiently sensitve method if only a small number of rabbit lung cells from the culture were capable of producing virus at any time and the block in the remainder is at or before this point.

The mechanism of the low grade persistent infection is not clear but it seems evident that there is a restriction of viral replication in rabbit cells, as has already been described in mouse cells (1). In addition, recent work by MOCARSKI and STINSKI (13) indicated that HCMV genome can persist over long periods if the virus is used to infect human fibroblasts at high multiplicities. Non-productive cells continued to grow and some of these spontaneously converted to productive viral infection. It was assumed that all sequences of the viral genome remained in the cells after infection and that viral gene expression was restricted in some way in the non-productive cells. The same situation may apply to the rabbit lung cells. Certainly, focal areas of infection could be seen even 28 days after infection and infectious virus was continously being produced at low level over this time. It may be that some host function is required to complete the viral replicative cycle which is seldom present, perhaps only being found in the rabbit lung cells at certain stages of the growth cycle. At other times the viral genome would become latent. It is still expressed, however, in 15 per cent of the cells as early antigens as shown by nuclear immunofluorescence up to one month after infection.

The question of latency of cytomegalovirus is an important one as this virus persists in man over many years, even in the presence of the host immune response. The cell type(s) and site(s) in the body of this persistence are uncertain but there may be a mechanism similar to that demonstrated here for rabbit lung cells, whereby cells are infected latently, with a small proportion of the population being capable of productive infection at any one time. The virus so produced could infect new cells and perpetuate the chronic state.

Aeknowledgments

We wish to thank the Cancer Research Campaign for funding this work.

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Authors' address: Dr. Mary Norval, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH 8 9AG, Scotland.

Received August 4, 1980

Printed in Austria

Gut, 1982, 23, 21–30

Lack of association of cytomegalovirus with adenocarcinoma of the colon

HELENA HART, W A NEILL, AND MARY NORVAL*

From the Department of Bacteriology, University of Edinburgh Medical School, Edinburgh

SUMMARY Tumour specimens from patients with adenocarcinoma of the colon or rectum were examined for the presence of cytomegalovirus (CMV), and specimens of normal mucosa from the same patients were studied in parallel. Frozen sections of 14 specimens were made and the presence of CMV mRNA assayed by *in situ* hybridisation using ^{3}H-labelled CMV-DNA as a probe. Nine of these sections were also tested for cytomegalovirus antigens by immunofluorescence. No viral nucleic acids or antigens were detected. In addition to these direct approaches, the specimens were disaggregated and 19 were successfully cultured in various media over several months without yielding virus on any occasion. Areas containing epithelial cells were found in some cultures, foci of bipolar cells in others, while, in several, fibroblastic cells predominated. To ensure that any virus-containing cells were not lost by this method, the disaggregated tumour and normal intestinal cells were directly co-cultivated and also fused with human embryo lung cells, which are permissive for cytomegalovirus replication. The resulting cultures were examined over two to three months for the presence of cytomegalovirus, and in no instance was virus found, despite attempted induction by iododeoxyuridine. Two fusion cultures became contaminated with cytomegalovirus, strain AD-169, which was being handled in the laboratory at the same time. The strain was identified by the pattern of viral DNA fragments produced by restriction endonuclease cleavage. Thus the accidental passage of virus in the heterokaryons did not alter its DNA and would further indicate the absence of any cytomegalovirus genomes in the adenocarcinoma cells.

Two groups have presented evidence recently that cytomegalovirus may be involved in adenocarcinoma of the colon. In the first place, colonic tissues from seven adenocarcinoma cases were analysed by cRNA-DNA hybridisation and over 50% contained more than two genome equivalents/cell.[1] Most control tissue obtained from normal colon and cases of Crohn's disease was negative. Secondly, several cases of adenocarcinoma of the colon were investigated by long-term culture of the tumour fragments.[2] Eight cell lines grew out, consisting mainly of fibroblastic cells, and cytomegalovirus was isolated from three of these. It was identified by electron microscopy, cytology of infected cells, and by immunofluorescence.

In both these situations, it is not known whether cytomegalovirus might be the aetiological agent or is merely reactivated from a latent state. It is of interest in this context that cytomegalovirus has been found in the gastrointestinal tract, and is claimed to be associated with chronic or debilitating diseases such as ulcerative colitis or intestinal ulcers.[3] Cytomegalovirus was isolated from three out of six cases of ulcerative colitis, and one out of four cases of regional enteritis by tissue culture.[4] In addition, cytomegalovirus inclusions were reported in macrophages and fibroblasts located in granulation tissues at the base of deep ulcerations in the colon and ileum and within endothelial cells in several cases of ulcerative colitis.[5] On the other hand, no virus was found after culture of cells from patients with Crohn's disease.[6]

Here we report our attempts to analyse further the association of cytomegalovirus with colon adenocarcinoma cells. In the first place, the results of culturing the tumour cells from 19 patients are given, also the results of co-cultivating and fusing these with human embryo lung cells which are permissive for human cytomegalovirus replication. The heterokaryons

*Address for correspondence: Dr M Norval, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

Received for publication 21 May 1981

produced from the fusions were treated with iododeoxyuridine to overcome the cellular control which might operate in such a situation.[7] The presence of cytomegalovirus was assayed by the observation of cytopathic effect and labelling with ^{3}H-thymidine. The cytomegalovirus isolates obtained were characterised by analysis of fragments produced by the restriction endonucleases EcoRI, Bgl II, and Hind III.

Secondly, 14 adenocarcinoma specimens were examined directly, as frozen sections, for cytomegalovirus antigens using immunofluorescence and for cytomegalovirus nucleic acids by *in situ* hybridisation. In no case was any evidence of cytomegalovirus found.

Methods

CLINICAL SPECIMENS

Surgically resected specimens of colon or rectum were obtained from the Royal Infirmary, Edinburgh, within a few minutes of being removed from the patient. Control specimens included pieces of normal colonic wall from the same patient, and others with Crohn's disease, ulcerative colitis, and diverticular disease. They underwent routine pathological examination, the diagnosis being based on this result. As soon as possible, and generally within two hours of the operation, small pieces of tumour (about 3 mm^3) and part of the normal colon were frozen by dropping into liquid nitrogen and were subsequently used to prepare 5 µm-thick frozen sections. These were stored at −20°C until required for immunofluorescence and *in situ* hybridisation studies. In addition, for culturing, similar small pieces were removed, finely minced and treated for one hour at 37°C in Earle's salt-based Eagle's medium containing 100 IU/ml gentamicin, 100 IU/ml mycostatin, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Soft tumours 'spilled out' cells during this time and these were used for culture, fusion, and co-cultivation experiments. Hard tumours and normal colon were digested with collagenase/dispase according to the manufacturer's instruction (Boehringer Mannheim) to obtain single cells.

The separated cells were used directly for culture in Eagle's medium containing antibiotics as above and supplemented with 10% fetal calf serum. They were seeded into Falcon flasks (Nunc) and observed for any morphological change over periods of two to three months. Latterly, a modification of this medium was used which contained D-valine.[8]

The disaggregated cells were also used for co-cultivation experiments after mixing with an equal number of human embryo lung cells. They were examined over the next two to three months, being subcultured three or four times in this interval.

CELLS

Human embryo lung cells were obtained by trypsinising human fetal lungs and seeding the cells in Eagle's medium supplemented with 10% fetal calf serum and containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Confluent fibroblastic monolayers were passed by trypsinisation and used between passage numbers 5–20, being grown in Eagle's medium with 5% newborn calf serum and being maintained in the same medium with 2% newborn calf serum. They were free of Mycoplasma contamination.

Two colon adenocarcinoma cell lines were obtained. HX18 line was kindly donated by Dr Courtenay who has described the growth and characteristics of these cells.[9] The other line came from Dr L K Woods and Dr G E Moore and has been described elsewhere.[10] Both cell lines grew in suspension, forming large clumps similar to those observed in fresh specimens of colon adenocarcinoma tumours. Both were grown in Eagle's medium with 10% fetal calf serum and antibiotics as for the human embryo lung cells above. They were contaminated with Mycoplasma species.

FUSION USING POLYETHYLENE GLYCOL

Initially, 'spill out' cells from adenocarcinoma specimens and disaggregated cells from normal colon were used directly for fusion. Latterly, the tumour cells were separated from the other cell types and non-viable cells by gravity sedimentation through 10 ml fetal calf serum for one hour at room temperature before fusion. The viable tumour cell clumps which formed were fused with human embryo lung cells using the method of Pontecorvo[11], which was slightly modified as already described and illustrated.[12] Various numbers of tumour and normal colonic cells were fused with 2×10^6 human embyro lung cells.

STIMULATION OF VIRUS PRODUCTION BY IODODEOXYURIDINE

Four weeks after the fusion, in the absence of a cytopathic effect, 20 µg/ml iododeoxyuridine (IUDR) was added to the medium for four days. It was then removed and the cultures observed as below for the presence of cytomegalovirus.

EXAMINATION OF CULTURES FOR THE PRESENCE OF CYTOMEGALOVIRUS

Cultured, co-cultivated, and fused colonic cells were examined over two to three months in the following ways: (1) microscopically for changes to

the cell sheet indicative of a cytopathic effect; (2) by labelling with 4 μCi/ml ^{3}H–thymidine for 48 hours and analysing the concentrated culture supernatant by sucrose density centrifugation;[13] (3) by anti-complement immunofluorescence[14] (ACIF) with cytomegalovirus seropositive human sera at a dilution of 1/20 and human complement, seronegative for cytomegalovirus. Anti-human C3 fluorescein tagged conjugate (Hyland) was used at 1/20 dilution.

The cytomegalovirus seropositive sera were tested by complement fixation and had titres of 2048. All contained antibodies to cytomegalovirus early and late antigens as judged by anti-complement immunofluorescence on human embryo lung cells infected with AD-169 two days post-infection and blocked with 40 μg/ml cytosine arabinoside (Sigma Chemical Company) (early), and on human embryo lung cells infected with AD-169 four days post-infection (late).

RESTRICTION ENDONUCLEASE ANALYSIS OF CYTOMEGALOVIRUS ISOLATES

A modification of the method of Kilpatrick *et al.*[15] and Huang (personal communication) was followed, using the DNA from infected cells rather than extracellular virus which may be difficult to obtain in large quantities from clinical isolates. Briefly, 4×10^{6} human embryo lung cells which had been infected with cytomegalovirus were labelled with ^{32}P (as orthophosphate in aqueous solution, carrier free, The Radiochemical Centre, Amersham) at 40 μCi/ml in low phosphate medium, supplemented with 2% dialysed newborn calf serum. After three days, the labelled cells were washed twice with 0·01M Tris-HCl buffered saline pH 7·4, and digested with 1 mg/ml pronase (Boehringer Mannheim) and 1% sodium dodecyl sulphate in lysis buffer (0·05M Tris-HCl, 0·01M EDTA, pH 8·0) for four hours at 37°. DNA was extracted using water-saturated phenol, precipitated with ethanol, and redissolved in buffer. It was purified by caesium chloride equilibrium density centrifugation. Fractions of density 1·71–1·73 g/m were pooled, dialysed overnight, precipitated with 2·5 volumes ethanol, and stored at −20°C until required. Then they were redissolved in 50 μl buffer containing 0·01M Tris-HCl, 0·001M EDTA, pH 7·4. Samples containing 10 000 counts per minute were digested with restriction endonucleases EcoR I, Bgl II, and Hind III (Millipore UK Ltd) in appropriate digestion buffers for two hours at 37°C. The restriction endonuclease fragments so produced were analysed on 1% agarose gels as described previously.[15]

IN SITU HYBRIDISATION

Cytomegalovirus DNA was prepared as already outlined.[16] Briefly, human cytomegalovirus, strain AD-169, was grown in human embryo lung cells and, after concentration by centrifugation of the culture supernatant, was purified by sucrose density gradient centrifugation. The band with density 1·20–1·23 g/ml was collected and re-purified on a second sucrose gradient. Viral DNA was extracted using SDS and proteinase K followed by banding in a 10–40% sucrose gradient. Viral DNA was extracted twice with phenol and precipitated with ethanol. It was finally banded by CsCl equilibrium density centrifugation, and the fractions with density 1·705–1·725 g/ml pooled, dialysed overnight, and the DNA precipitated with ethanol. The precipitate was dissolved finally in TES buffer (0·05M Tris-HCl, 0·15M NaCl, 0·005M EDTA, pH 7·4) at a concentration of 50 μg/ml.

Nick-translation was then carried out,[17] the reaction mixture containing all four nucleotide triphosphates labelled with tritium (Amersham) and incubation continuing for five hours. Cpm/μg nucleic acid after separation on a Sephadex G50 column was around 10^{7}.

In situ hybridisation was carried out on frozen sections of colon which were fixed in ethanol: acetic acid, 3:1, for 10 minutes at −20°C just before use, according to the method obtained from Rapp's laboratory.

IMMUNOFLUORESCENCE

The technique of amplified direct immunofluorescence (AMDI) as described by Schmitz and Kampa[18] was used for the detection of cytomegalovirus antigens on frozen sections of tumour and normal colons. Two human sera were selected with antibodies to cytomegalovirus early antigens. Both had complement fixation titres of 1024 and, by ACIF, both stained only nuclei from cytomegalovirus-infected human embryo lung cells, one giving mainly a general nuclear staining and the other showing granular staining on early-infected nuclei. No non-specific antinuclear staining was present. These sera were labelled with fluorescein iso-thiocyanate, isomer I (FITC, Fluka 46952).[19] For the AMDI test, the frozen sections were pre-incubated with human sera, negative for cytomegalovirus, at a dilution of 1/10 for one hour at room temperature. This was followed by the FITC-labelled human sera at a dilution of 1/100, then rabbit anti-FITC serum at a dilution of 1/160, which had been kindly donated by Drs Schmitz and Kampa. Finally, anti-rabbit IgG labelled with FITC (Wellcome Laboratories) was used at 1/16.

Results

CULTURE OF TUMOUR CELLS

Cell cultures were set up from tumour specimens of 35 patients who were diagnosed histologically as having adenocarcinoma of the colon or rectum.

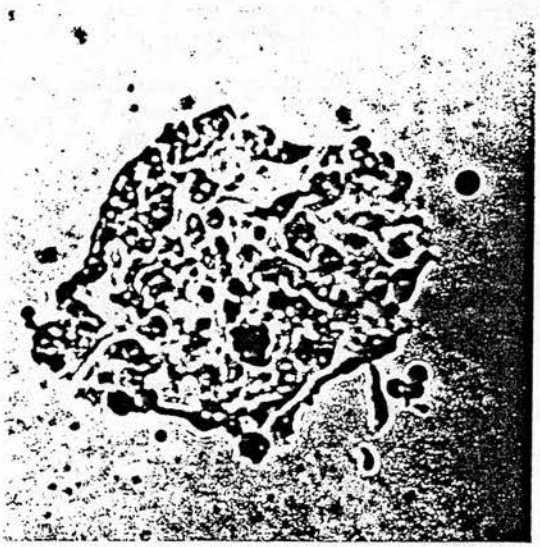

Fig. 1 *Epithelial cells obtained on culture of colonic adenocarcinoma specimen. x 211, phase contrast. (In this and the following figures original magnifications are given.)*

Fig. 2 *Island of epithelial cells derived from colonic adenocarcinoma after co-cultivation with human embryo lung cells. x 211, phase contrast.*

Cell growth was obtained in 19 of these. Areas of epithelial cells were observed in seven cultures, occasionally only when co-cultivated with human embryo lung cells. These are shown in Figs. 1 and 2. They appeared after two to three days in culture and persisted for two weeks to three months, dividing several times in this interval. However, attempts to subculture them were always unsuccessful, so that no lines of epithelial cells derived from adenocarcinomas have been established. The use of medium containing D-valine did not increase the number of epithelial cells obtained, promote their growth, or help maintain them on subculture.

Cultures containing discrete foci of bipolar cells, which have been described by Leibovitz *et al*,[20] were sometimes seen and are demonstrated in Fig. 3. These cells also failed to grow on subculture.

Cells with characteristic fibroblastic morphology grew out from seven specimens and are shown in Fig. 4. These could be successfully subcultured at

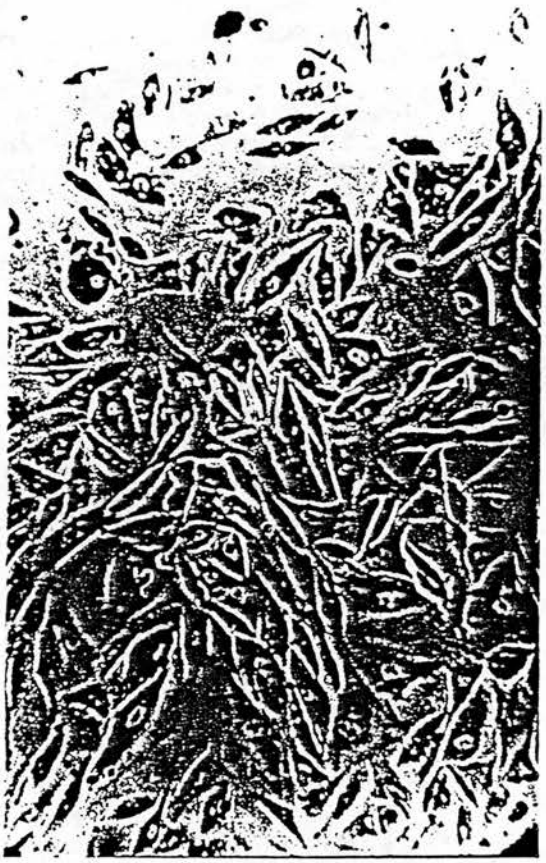

Fig. 3 *Bipolar cells obtained on culture of colonic adenocarcinoma specimen. x 825, phase contrast.*

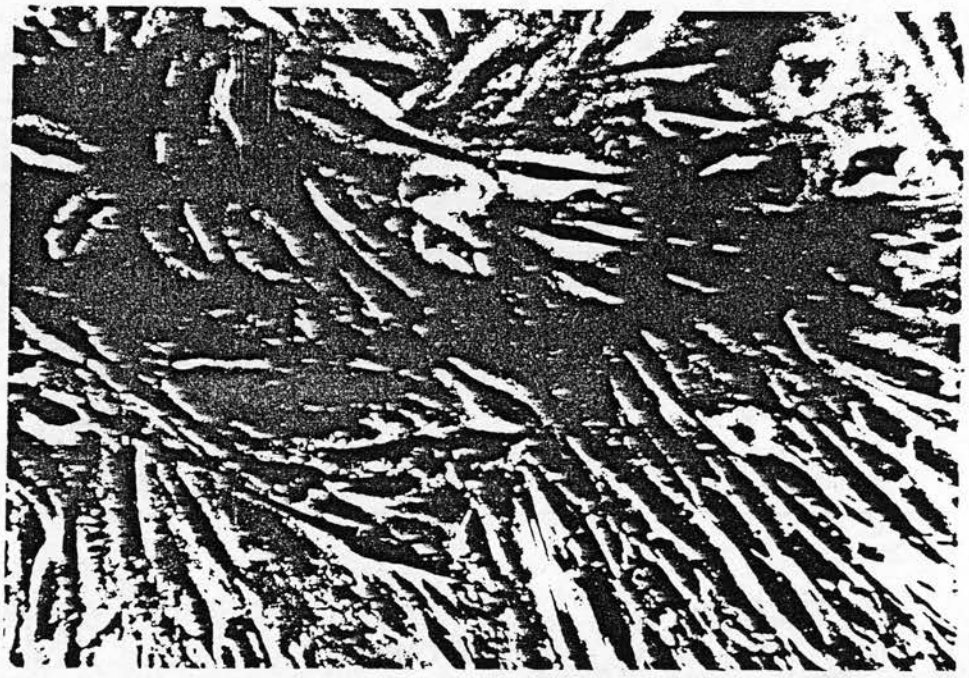

Fig. 4 *Fibroblastic cells obtained on culture of colonic adenocarcinoma specimen. x 825, phase contrast.*

two-weekly intervals for periods of up to four months. In one case, a mixture of fibroblasts and epithelial cells was obtained.

All cultures were observed microscopically every week, and at no time was a cytopathic effect apparent.

In addition to culturing cells from tumour specimens, pieces of normal intestinal mucosa from the same patient were used. All 17 cultures set up from this source grew as fibroblasts, identical with those fibroblastic cells obtained from the seven adenocarcinomas described above. Again, no cytopathic effect was apparent over a three month period of observation.

A summary is given in Table 1 of the types of cells which were obtained on culture from the carcinoma specimens, together with the degree of differentiation of the tumour and the Dukes's stage.

Table 1 *Details of cell type obtained on culture of colonic or rectal carcinomas, degree of differentiation of tumour, and Dukes's stage*

Degree of differentiation	*Dukes's stage B*			*Dukes's stage C*		
	Well	*Moderate*	*Poor*	*Well*	*Moderate*	*Poor*
Cell type:						
Fibroblastic	0	1	2	1	1	2
Epithelial	0	4	0	1	2	0
Bipolar	2	1	0	0	2	1
No growth	0	7	6	0	2	1

CO-CULTIVATION

Equal numbers of viable cells from adenocarcinoma of the colon or rectum and cells from pieces of normal colon were mixed with human embryo lung cells and used to seed Falcon flasks. Specimens from six patients were used, three of Dukes's stage B moderately differentiated, one of stage B poorly differentiated, one of stage C well differentiated, and one of stage C moderately differentiated.

Initially, there was a cytotoxic effect on the human embryo lung cells, so that, after two or three days in culture, some were detached and others more rounded and granular than normal. The ones which survived gradually grew out into monolayers, this taking about a month. Some colonic cells with a different morphology from human embryo lung cells persisted over this time also. The cultures were split at about monthly intervals and observed microscopically over two to three months for cytopathic effect. None was seen, although sometimes the cells looked granular and did not grow as quickly as normal. Labelling of the cultures with ^{3}H-thymidine followed by sucrose density gradient centrifugation of the concentrated culture supernatant did not reveal radioactive material with the density of cytomegalovirus.

FUSION

Paired samples of fresh tumour and normal colonic

Table 2 *Details of specimens used in fusion experiments, together with viable counts of tumour cells and observations of cytopathic effect (CPE) before and after treatment with IUDR*

Number	*Diagnosis*	*Degree of differentiation of tumour*	*Dukes's stage of carcinoma*	*Number of viable cells obtained for fusion*	*Comments on tumour cell suspension before fusion*	*CPE*	*CPE after IUDR treatment*
790004	Rectal carcinoma	Moderate	B	T 2×10^6	20% viable	—	—
				N 2×10^6		—	—
790020	Rectal carcinoma	Moderate	B	T 4×10^6	Large numbers	++	○
				N 4×10^5	of polymorphs	+++	○
790021	Colon carcinoma	Moderate with foci of poorer differentiation	B	T 6×10^6	10% viable	+++	○
				N 10^6		—	○
790028	Colon carcinoma	Moderate	B	T $2{\cdot}5\times10^5$	80% viable	—	—
				N 10^6	clumps	—	—
790043	Colon carcinoma	Moderate	A	T $2{\cdot}4\times10^5$	80% viable	—	—
				N $1{\cdot}5\times10^6$	clumps	—	—
790046	Colon carcinoma	Moderate	B	T 2×10^6	80% viable	—	—
				N 4×10^6	clumps	—	—
790083	Colon carcinoma	Moderate	B	T 10^6	90% viable	—	—
				N 10^6		—	—
790088	Colon carcinoma	Poor	C	T 5×10^5	90% viable	—	—
				N 2×10^6		—	—

Tumour cells from specimens 790028-790088 were purified by gravity sedimentation through fetal calf serum before fusion. T: cells from tumour. N: cells from normal mucosa of same patient. CPE: cytopathic effect. +++, ++, +: degrees of CPE. —: negative result. ○: not done or not tested.

mucosal cells were fused with human embryo lung cells. The diagnosis of patients from whom the specimens were obtained is given in Table 2. Initially, it was found that the disaggregated tumour contained a mixture of cells whose viability ranged from 10–20%. Gravity sedimentation through a layer of fetal calf serum increased the purity and number of viable cell clumps to 80–90% and these were used latterly for the fusion (Table 2). The cultures of fused cells were observed for periods of two months. On two separate occasions a cytopathic effect was observed in the heterokaryons from two patients—namely, 790020 and 790021—five and six weeks respectively after the fusion.

These isolates proved to be cytomegalovirus by anti-complement immunofluorescence and by labelling with ^{3}H-thymidine, followed by sucrose density gradient centrifugation, which revealed particles of the same density as that of cytomegalovirus.

They were further analysed by restriction endonuclease cleavage of the viral DNA. The fragments produced showed complete identity with AD-169 strain of cytomegalovirus (Fig. 5). The virus labelled 790021T had an extra band of high molecular weight which was thought to represent incomplete digestion rather than any real difference in the pattern. Strain AD-169 was being used in the laboratory at the same time as these cultures. Great care, including the use of a separate room, was taken to handle the clinical specimens away from this virus, but it had to be assumed that contamination had occurred on these two occasions, as it is hardly possible that both strains isolated should have the characteristics of AD-169.

No viruses were isolated from subsequent fusions. This was judged by (1) absence of cytopathic effect over a period of two months, even after attempted stimulation of virus production with IUDR; (2) labelling with ^{3}H-thymidine two months after the fusion to ensure that the absence of cytopathic effect also meant the absence of productive viral infection; and (3) anti-complement immunofluorescence which was negative on both the unstimulated and IUDR-stimulated heterokaryons using three specially selected high titre sera with antibodies to cytomegalovirus early and late antigens.

In addition to the fusion attempts with freshly isolated tumour cells, the two adenocarcinoma cell lines were fused with human embryo lung cells. No virus was rescued from these even after IUDR treatment. Also both the original colonic lines and their heterokaryons did not express the cytomegalovirus early or late antigens as tested by anti-complement immunofluorescence.

IN SITU HYBRIDISATION

The patients from whom the specimens were obtained for *in situ* hybridisation are shown in Table 3 along with the pathological diagnosis. In each case a piece of tumour was used to make frozen sections and also a piece of normal intestine away from the site of the tumour. These sections, were subjected to *in situ* hybridisation to assay for cytomegalovirus mRNA within the cells using ^{3}H-labelled DNA of cytomegalovirus as a probe. As a positive control, human embryo lung cells were infected with cytomegalovirus and fixed two days after infection. When developed, after hybridisation and autoradiography, the infected cells showed an average of over 100

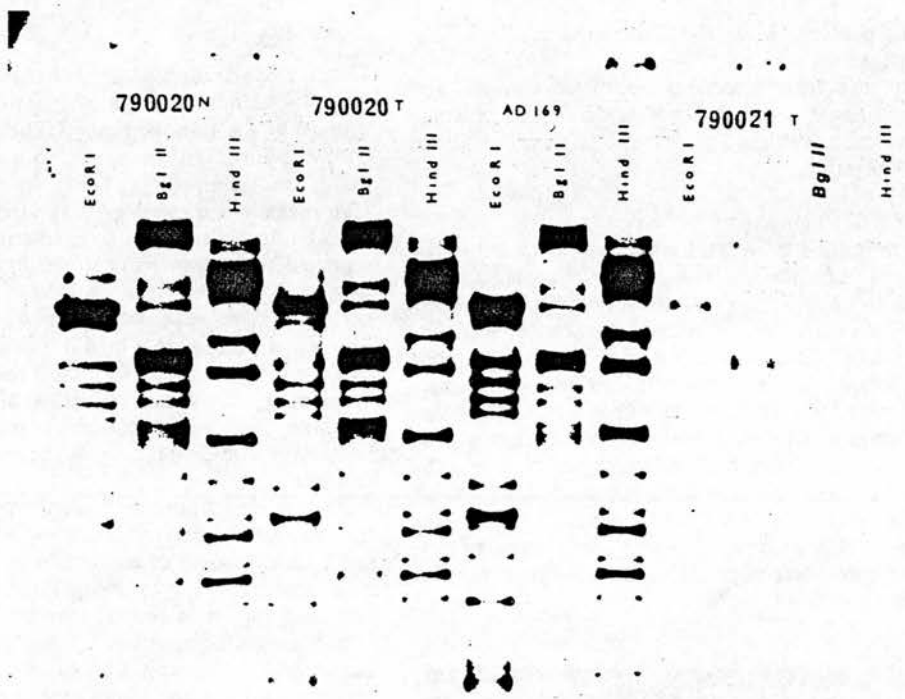

Fig. 5 *Restriction endonuclease patterns of DNA from cytomegalovirus strain AD-169, and cytomegalovirus passaged in heterokaryons of human embryo lung cells fused with normal (N) and tumour (T) colonic cells of patient 790020, and tumour (T) colonic cells of patient 790021. The viral DNA was digested with EcoRI, BglII, and HindIII endonucleases.*

Table 3 *Specimens used for* in situ *hybridisation with CMV ^{3}H-DNA as probe, and for amplified direct immunofluorescence with human CMV antisera*

Number	*Diagnosis*	*Sex, age (yr)*	*Dukes's stage*	*Degree of differentiation*	*In situ hybridisation*	*Immuno-fluorescence*
790020	Rectal adenocarcinoma	F 57	B	Moderate	Negative	Not done
790082	Diverticular disease	F 70			Negative	Not done
790083	Colonic adenocarcinoma	F 84	B	Moderate	Negative	Negative
790084	Colonic adenocarcinoma	F 74	C	Poor	Negative	Negative
790111	Colonic adenocarcinoma	F 73	C	Moderate	Negative	Not done
790112	Colonic adenocarcinoma	M 57	C	Poor	Negative	Not done
80037	Caecal adenocarcinoma	M 50	B	Poor	Negative	Negative
80045	Colonic adenocarcinoma	F 83	C	Moderate	Negative	Negative
80046	Rectal adenocarcinoma	M 76	B	Moderate	Negative	Negative
80054	Caecal adenocarcinoma	M 75	B	Well	Negative	Not done
80055	Caecal adenocarcinoma	F 69	C	Moderate	Negative	Negative
80067	Colonic adenocarcinoma	M 59	B	Poor	Negative	Negative
80084	Caecal adenocarcinoma	F 63	B	Moderate	Negative	Not done
80085	Caecal adenocarcinoma	F 81	C	Poor	Negative	Not done
80105	Caecal adenocarcinoma	M 75	—	Poor	Not done	Negative
80106	Colonic adenocarcinoma	F 79	C	Moderate	Not done	Negative

grains per cell, while the uninfected cells had less than 2 grains per cell.

For the frozen sections, both of tumour and normal tissue, in no case was any cell seen in which there were more than 2 grains, and all were concluded to be negative.

IMMUNOFLUORESCENCE

The technique of AMDI was used to examine the sections of human tissue for cytomegalovirus antigens as the background staining obtained by ACIF and indirect immunofluorescence was unacceptable. This might be due to complement receptors,[21] or to antibody-antigen complexes present on the surface of the mucosal epithelial cells. In common with many other groups, attempts to produce animal sera with sufficient amounts of antibody to early antigens were not successful. When AMDI was used on human embryo lung cells infected with cytomegalovirus, the pattern of immunofluorescence appeared as a uniform staining of nuclear antigens (Fig. 6).

Fig. 6 *AD-169 infected human embryo lung cells (early antigens) strained by amplified direct immunofluorescence (AMDI) showing a uniform staining of nuclear antigens.*

Frozen sections from nine paired specimens of adenocarcinoma and normal gut mucosa were screened by AMDI as shown in Table 3 and all were negative. Occasionally, immunofluorescence was seen in the normal mucosa adjacent to the tumour. This seemed to be due to the cross-reactivity of batches of FITC anti-rabbit IgG with human IgG, and could be eliminated by selecting non-cross-reactive conjugates.

Discussion

Cytomegalovirus has been isolated from several cases of adenocarcinoma of the colon by long-term culture of the tumour tissue.[2] The cells which grew out were fibroblastic in appearance with less than 1% being epithelial, these being lost on subculture. Cytomegalovirus was not apparent until several weeks after initiation of the culture. In the work reported here, long-term cultures of adenocarcinoma tissues did not yield cytomegalovirus on any occasion. The cells which grew were sometimes fibroblastic, although a medium containing D-valine was used in an attempt to inhibit these.[8] Small areas containing tightly packed epithelial cells were seen in about 30% of the cultures a few days after the initiation; these were subsequently lost on subculture.

As cells containing cytomegalovirus might not be the ones which survived and grew in culture, we also used the techniques of co-cultivation and fusion with cells permissive for cytomegalovirus replication. It has been shown in several systems that, if a whole viral genome is present in a transformed cell, it can be rescued in this way. One example is the lymphoblastoid Raji cell line which contains 50–60 copies of Epstein Barr virus DNA but does not produce infectious virions unless fused with D-98 (a derivative of HeLa cells) and induced with IUDR.[22]

Of the Herpes viruses, the transforming ability of cytomegalovirus is least well known, although it has been shown to have the potential to transform in several *in vitro* systems[23-25]. However, the low numbers of successful examples of such transformations might indicate that an event of tumour promotion is required in addition to the initial viral stimulation of cell DNA.[26] It is possible, for example, that human cytomegalovirus could be responsible for transformation of the mucosal cells of the epithelial lining of the large bowel and that this process is promoted by dietary factors or bile salts. Transformation by cytomegalovirus of epithelial cells has not been reported *in vitro*. The virus seems to undergo a low semi-permissive type of replication in human epithelial amnion cells,[27] human lung epithelial cells,[28] and human ecto- and endocervical cells.[39]

Both co-cultivation and fusion experiments did not yield infectious cytomegalovirus even after prolonged culture. In addition, treatment with IUDR, which has been shown to be necessary for activation of cytomegalovirus from a latent state in heterokaryons of non-permissive cells,[7] did not induce any virus. On two occasions accidental contamination with AD-169 strain of cytomegalovirus occurred, despite great care being taken in the

handling of these long-term cultures. It was of interest, however, that the fragments produced by restriction endonuclease cleavage of viral DNA were identical for the virus grown in human embryo lung cells and that passaged in the adenocarcinoma tumour cell-human embryo cell heterokaryons. This does not suggest a recombination with other cytomegalovirus genetic material which might be present in the colonic cells.

Of course, it is possible that either the whole genome is not present in the adenocarcinoma cells or that it cannot be rescued by such culture methods. The latter is true of another transforming virus, Adeno type 12, which recently has been shown to induce rat brain tumors *in vivo* and to integrate the entire viral genome in the host cell. However, no infectious virus could be obtained from subsequent fusions or co-cultivations of the tumour cells with permissive cells.[30]

Therefore, two more direct approaches were used during this work—firstly, AMDI to detect cytomegalovirus antigens on frozen sections of adenocarcinoma and normal intestinal specimens, and secondly *in situ* hybridisation to detect cytomegalovirus mRNA.

Looking for viral antigens by an AMDI test has proved a good approach where the availability of specific animal antiviral serum is limited by the restriction of viral replication to human cells, as is the case with cytomegalovirus. Until a mixture of monclonal antibodies detecting a range of early and late cytomegalovirus antigens is available, antibody-positive human sera are the least cross-reactive reagents with human biopsies. No immunofluorescence was found using the two human sera selected, although they contained antibodies to early and late cytomegalovirus antigens, and thus no cytomegalovirus antigens were shown to be present in the tumour or normal intestinal cells.

In situ hybridisation has proved a useful technique in many systems to search for the presence of viral nucleic acids in cells where there is no productive infection—for example, it has been used to detect *Herpes simplex* type 2 mRNA in sections prepared from frozen biopsies of patients with cervical dysplasia.[31] This method has the considerable advantage of picking out perhaps only one or two cells in the section which might contain viral mRN and, in addition, there should be amplification of any viral information by assaying the mRNA rather than DNA. No hybridisation was found in the intestinal tumour or normal sections with cytomegalovirus ^{3}H-DNA as probe. Huang and Roche used another hybridisation technique—that of membrane cRNA-DNA hybridisation, which detects viral DNA extracted from the tumour specimens—and found that more than 50% of patients with adenocarcinoma of the colon had greater than 2 genome equivalents of CMV/cell.[1] However, very recently, this work has not been corroborated using a DNA–DNA reassociation kinetics test which had an estimated sensitivity of 0·3 genomes equivalents per cell. No cytomegalovirus DNA was detected in biopsy specimens from seven patients with adenocarcinoma of the colon.[32] Such a method has given positive results in examining tumour cells of patients with Kaposi's sarcoma cytomegalovirus genomes.[33] Thus, there is considerable doubt as to whether the colonic adenocarcinoma cells contain any cytomegalovirus DNA, although it may not be possible to detect a smaller (< 0·3 genome equivalents) fragment of viral DNA. However, a smaller fragment amplified as mRNA or specific viral protein could be detected by *in situ* hybridisation and AMDI immunofluorescence.

We conclude, therefore, that cytomegalovirus is unlikely to contribute to the maintenance of the transformed phenotype of the adenocarcinoma cells and that there is no localised cytomegalovirus reactivation contiguous to the tumour, a situation observed in metastatic nasopharyngeal carcinoma.[34]

We wish to thank Mr I McLeod and his theatre staff at the Royal Infirmary of Edinburgh for their very helpful co-operation in this work. Also we are grateful to Dr H Gilmour for drawing up the case histories of patients and for doing the histological examination of specimens. We thank Dr E-S Huang for showing Dr H Hart the technique of restriction endonuclease analysis of cytomegalovirus in his laboratory in Chapel Hill, North Carolina. Grant support for this work was given by the Cancer Research Campaign.

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Gynecol. obstet. Invest. *14:* 300–308 (1982)

Lack of Association of Cytomegalovirus Antibody Level with Carcinoma of the Uterine Cervix

Helena Hart[a], *Anthea Springbett*[b], *Mary Norval*[a]

[a] Department of Bacteriology, University of Edinburgh Medical School;
[b] MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh, UK

Key Words. Cytomegalovirus antibodies · Cervical carcinoma and dysplasia · ELISA, Anti-complement immunofluorescence · Neutralisation

Abstract. Serum samples were collected from 199 patients attending a gynaecological clinic, colposcopy specimens being taken at the same time for routine pathology of paraffin sections which ranged from normal, through the degrees of dysplasia to carcinoma in situ. Other parameters noted were age, number of pregnancies and time since last pregnancy. When level of antibody to cytomegalovirus in each serum sample was tested by ELISA, anti-complement immunofluorescence and neutralisation, no correlation was found between antibody titre and pathological diagnosis. The only interdependence seen was that, as the age of the patients increased, so did the frequency of sero-positivity to cytomegalovirus. Also in the group studied the frequency of carcinoma in situ was not age dependent.

Introduction

The association of cytomegalovirus (CMV) with carcinoma of the uterine cervix has been tentatively suggested both by some sero-epidemiological surveys and, more recently, by virus isolations. In addition, CMV has been found in semen as well as the urogenital tract, may be associated with venereal diseases and has been demonstrated to have oncogenic potential [*Melnick* et al., 1974]. *Vestergaard* et al. [1972] reported a significantly higher level of antibody to CMV in sera from patients with newly diagnosed cervical carcinoma compared to matched controls, although this was not found by *Fuccillo* et al. [1971] when looking at antibody levels in patients prior to the development of carcinoma in situ. *Pasca* et al. [1975] found

antibodies to CMV more frequently in sera of women with atypia than in women with cervical disorders other than atypia and in controls. Lastly, in 1978, *Melnick* et al. isolated CMV from two cell cultures derived from cervical biopsies of patients with advanced carcinoma.

The serological surveys mentioned above were performed using a complement fixation test and newer methods such as enzyme-linked immunosorbent assays (ELISA) and anti-complement immunofluorescence (ACIF) known to be more sensitive [*Booth* et al., 1979], as well as by measuring different classes of antibody. Thus we decided to take a group of women with cervical disorders ranging from mild, moderate and severe dysplasia to carcinoma in situ and to measure their circulating antibody titre to CMV, using three methods, namely ELISA, ACIF and neutralisation. These results were then correlated with their age, parity, time since last pregnancy and histological diagnosis of colposcopy specimens taken on the day of serum sampling

Materials and Methods

199 sera and colposcopy specimens were collected over a period of 2 years from patients attending the Gynaecology Outpatients Department at the Royal Infirmary of Edinburgh. These patients all had abnormal cervical smears and were referred for further investigation. The colposcopy specimens were paraffin-embedded for routine pathology examination and the patients subsequently divided into six groups on the basis of this result, namely: (1) normal, 18 patients; (2) abnormal, but no dysplasia, 25 patients; (3) mild dysplasia, 14 patients; (4) moderate dysplasia, 38 patients; (5) severe dysplasia, 63 patients, and (6) carcinoma in situ, 41 patients. In cases where different degrees of dysplasia were reported in specimens from the same patient, the most severe degree was used for placement in the group. The patients were not matched individually but additional parameters of age, parity, date of last delivery or termination of pregnancy were noted and their distribution compared for each group.

The tests used for detection of antibodies to CMV were ELISA, ACIF and neutralisation. These were carried out in batches of 35 samples and always included duplicates from other batches.

ELISA

This was carried out as described by *Booth* et al. [1979]. Titres ranged from < 10 to 12,800.

ACIF

This was designed to detect antibodies to early CMV antigens, using the method of *Kettering* et al. [1977] in outline. Human embryo fibroblasts were infected with CMV strain AD-169, in the presence of 40μg/ml cytosine arabinoside and harvested 48 h after

infection. 2×10^4 cells/spot were placed on multispot slides (Hendley-Essex), allowed to dry in air and fixed in cold acetone for 10 min. Doubling dilutions of sera starting at 1/10 were allowed to react for 1 h at 37 °C, human complement (serum sero-negative for CMV) was used at a dilution of 1/4 overnight at 4 °C, and anti-human complement C3 labelled with FITC (Hylland Division of Travenol Laboratories) at 1/40 reacted for 1 h at 37 °C. Titres ranged from <10 to 160.

Neutralisation

Serum samples were inactivated at 56 °C for 30 min, then doubling dilutions were allowed to react for 30 min at 37 °C with equal volumes of CMV strain AD-169 containing 50 PFU/10 µl. 10 µl samples were assayed for any residual viral activity using human embryo fibroblasts grown on microtitre plates (Sterilin). The titre was expressed as the highest dilution of serum producing a 50% or greater reduction in the plaque count and ranged from <2 to 128.

Statistical Analysis

This was carried out by the Statistical Unit at the Western General Hospital, Edinburgh, using the Health Sciences Computing Facility of the University of California. Two-way contingency tables of serological results by each of the three methods showed independence between batches of sera. Four-way contingency tables of serological results and pathological diagnosis were drawn up, also four-way contingency tables of pathological diagnosis, age, parity and time since last delivery or termination of pregnancy. ANOVA of serological results by pathological diagnosis and age was calculated using no transformation and log transformation. Three age groups were made for this, 18–27 years, 28–37 years and over 38 years.

Results

The distribution of the patients' parameters, age, parity and time since last delivery or termination of pregnancy was calculated in each of the six groups. Using four-way contingency tables, there was no significant difference between any of these factors.

Initially, the results of the three antibody tests to cytomegalovirus, ACIF, ELISA and neutralisation were divided into positive and negative, taking as negative titres of $\leqslant 10$ in the ACIF test, $\leqslant 100$ in the ELISA test and $\leqslant 2$ in the neutralisation test; titres greater than these were taken as being positive. A four-way contingency table analysis of these results showed independence with respect to the pathological diagnosis of the patients. Thus, for example, there was no correlation of the presence of CMV antibody in any of the three tests with the carcinoma group as might have been expected if CMV was associated with this condition. There was a strong interdependence of the three tests used so that if one

was positive, the other two had a very significant tendency to be positive also.

In addition to the division of serological results into positive and negative, a comparison was made of the means of titres in the three tests for the various groups of patients. For this, four groups were made: normal/abnormal but no dysplasia, mild/moderate dysplasia, severe dysplasia and lastly, carcinoma in situ. The results are illustrated in figure 1. It may be seen that no group had a significantly higher mean titre than any other.

Taking each of the patients' parameters in turn and correlating these with the antibody results, the only interdependence demonstrated was the significant increase in sero-positive patients as their age increased. An example of this is given in table I for the ELISA results, dividing the patients arbitrarily into four age groups.

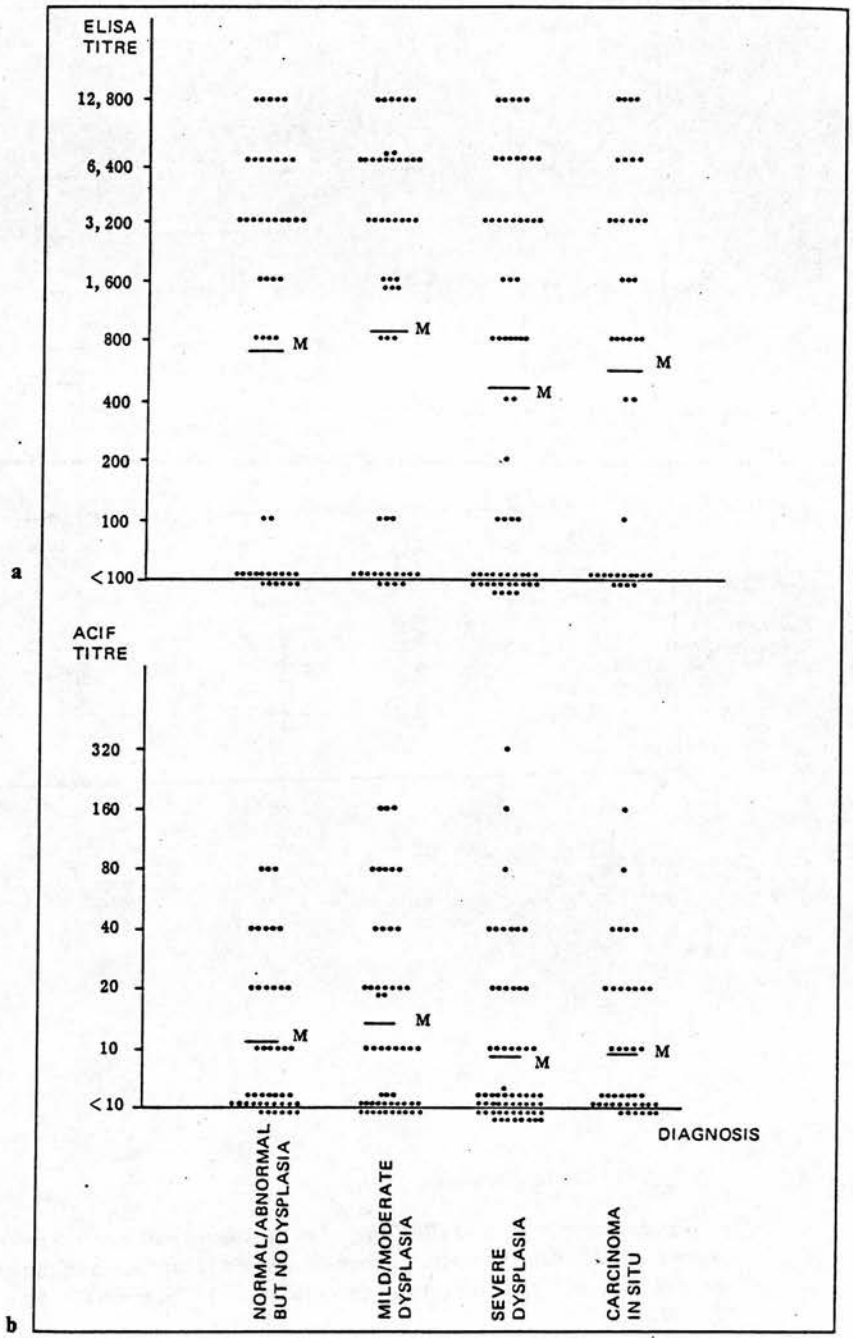
In addition, it was also noted that, in the groups studied, there was no increase in the frequency of cervical carcinoma as the age of the patients increased. These results are shown in table II.

Table I. Number of patients, divided into groups by age, who were sero-positive for CMV as measured by the ELISA test

Age of patient years	ELISA titres		Sero-positive for CMV %
	$\geqslant 200$	$\leqslant 100$	
18–25	20	19	51.3
26–33	56	45	55.4
34–41	28	14	66.7
>41	15	1	93.8

Table II. Number of patients, divided into groups by age, who were diagnosed as having carcinoma in situ

Age of patient years	Number with carcinoma in situ	Total number	Carcinoma in situ %
18–25	9	39	20.8
26–33	19	101	18.8
34–41	11	42	26.2
>41	2	16	12.5


a
ELISA TITRE
12, 800
6, 400
3, 200
1, 600
800
400
200
100
<100
M
b
ACIF TITRE
320
160
80
40
20
10
<10
DIAGNOSIS
NORMAL/ABNORMAL BUT NO DYSPLASIA
MILD/MODERATE DYSPLASIA
SEVERE DYSPLASIA
CARCINOMA IN SITU

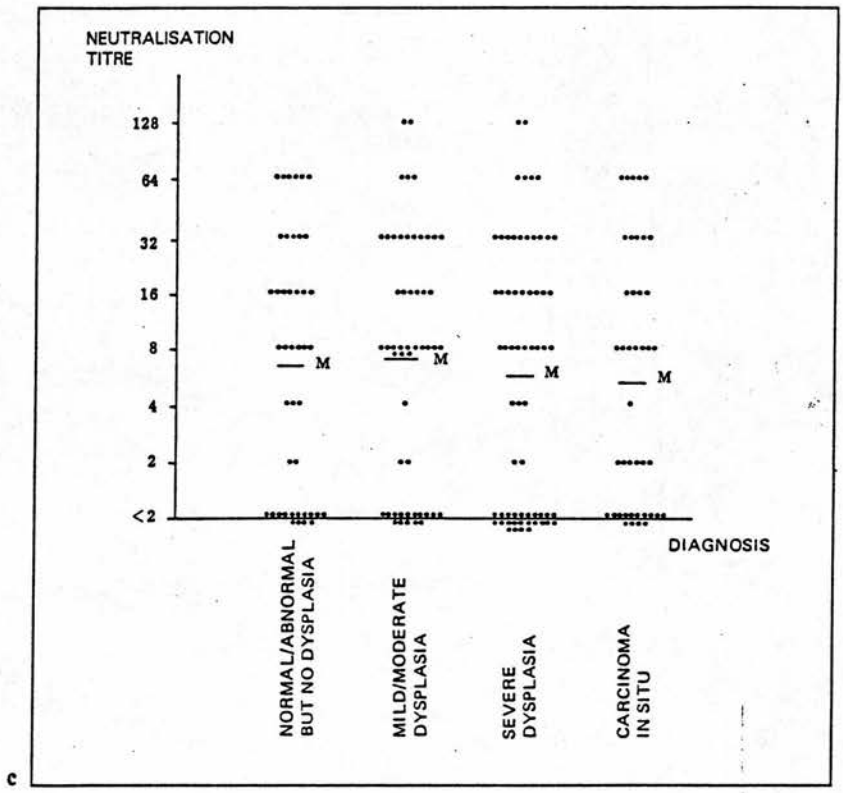


Fig. 1. Antibody titres to CMV in groups of patients with normal/abnormal but no dysplasia, mild/moderate dysplasia, severe dysplasia and carcinoma in situ of the cervix, measured by ELISA (a), ACIF (b) and neutralisation (c). The mean (M) for each group is indicated.

Discussion

Several viruses have been suggested as being implicated in the aetiology of cervical carcinoma, the most common being *Herpes simplex* type 2, and CMV. *Thomas and Rawls* [1978] using complement fixation found a higher level of antibodies to *H. simplex* in patients with carcinoma in situ or severe dysplasia but not in milder dysplasia. More recently, *McDougall* et al. [1980] failed to detect mRNA of this virus in specimens of invasive carcinoma, although they could find it in cells undergoing pre-malignant change. Also *Fenoglio* et al. [1980] were able to detect Herpes early antigens in early cervical intraepithelial neoplasia by immunoperoxidase staining but this was lost as the condition proceeded to carcinoma in situ. It is obviously very difficult to differentiate an initiating agent from one which is present, as Herpes is, in the urogenital tract in a considerable number of people and the same applies to CMV. This virus is often found in cervical secretions; in a study by *Waner* et al. [1977] about 8% of patients with suspected venereal disease were excreting CMV and there was local production of antibodies to CMV in over half of them.

During the course of this work, three methods were employed to detect antibodies to CMV. The ACIF test was designed to measure antibodies to early viral antigens, while the other two measured presumably predominantly late antigens. The three tests were found to be interdependent to a very large extent and not to vary significantly with batches of sera. Of the patients' parameters, age, parity, time since last pregnancy and pathological diagnosis of cervix, no correlation was found with antibody titre to CMV, except, as the patients got older, the number who were sero-positive increased. So, no serological evidence is available from this study to associate CMV with carcinoma of the cervix or cervical dysplasia. Also, there was no suggestion of differential response of these groups to the early CMV antigens. We were interested in the number and timing of pregnancies as CMV is well-known to reactivate during pregnancy but there was no interdependence of this factor and antibody titre. It was of interest that, when age and pathological diagnosis were compared, there was a similar number of patients in the younger age groups with cervical carcinoma as in the older age groups. The same result was reported by *Mendis* et al., [1979] who found 14% of their patients with carcinoma to be 30 years or younger, and would further indicate that national screening programmes should include all sexually active women over 20 years.

In addition to the serological status of patients with cervical disorders, we were also interested in their cell-mediated immunity to CMV and are now measuring this additional parameter.

Acknowledgements

We wish to thank Dr. *G. Smart,* Dr. *J. Livingstone* and Sister *Aitken* of the Gynecology Department of the Royal Infirmary of Edinburgh for their co-operation in providing the clinical material used in this study, also Mrs *M. Murray* for collecting details of the patients, and the staff of the Department of Pathology for marketing the pathological results of colposcopy specimens available to us. This study was funded the Cancer Research Campaign.

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Received: September 14, 1981; accepted: December 17, 1981

Helena Hart, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG (UK)

Gynecol. obstet. Invest. *18:* 122–128 (1984)

0378–7346/84/0183–0122$2.75/0

Natural Killer Cell Activity in Patients with Abnormalities of the Uterine Cervix

W. Neill, M. Norval

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, UK

Key Words. Natural killer cells · Killer cells · Cervical dysplasias · Cytomegalovirus

Abstract. Peripheral blood lymphocytes from 105 patients with various degrees of cervical dysplasia (normal/mild, moderate and severe) up to carcinoma in situ were assayed for natural killer cell activity against K562 cells and Chang liver cells in vitro. No difference was found between the natural killer cell activities of any of the groups of patients studied. In addition, the K cell activity of the patients' lymphocytes was assayed in an antibody-dependent cellular cytotoxicity assay on Chang liver cells with anti-Chang cell antiserum. No difference was found between patient groups. There was no correlation between the presence of antibodies in the patients' serum to cytomegalovirus measured by anti-complement immunofluorescence and increased natural killer cell activity.

Introduction

While it is generally accepted that T lymphocytes play a major role in specific tumour immunity, there is now evidence that natural killer (NK) cells may provide early resistance against viral infections, tumour growth or metastasis [1–3]. NK cells are lymphoid-like cells which circulate in the peripheral blood and possess the ability to lyse both tumour cells and virus-infected cells spontaneously without pre-sensitization. In that respect NK cells differ from classical cytotoxic T lymphocytes (CTL) whose killing is restricted by the major histocompatibility complex (MHC). NK cell activity has been assayed in vitro by lysis of K562 cells which represent a myeloid leukaemia cell line [4]. When NK cells were partially purified from peripheral blood or spleen by centrifugation through Percoll gradients, most activity was associated with the cell population containing large granular lymphocytes (LGL) [5].

Low or negligible NK activity was initially demonstrated in beige (bg) mice [6] and subsequently in a number of human disease states such as Chédiak-Higashi syndrome [7], severe combined immunodeficiency [8] and malignancy [9–12].

Antibody-dependent cell-mediated cytotoxicity (ADCC) is mediated by killer (K) cells, which, in common with NK cells, bear

receptors for the Fc portion of IgG. Target cells must be pre-coated with specific antibody for induction of K cell lysis, although it would appear that NK and K cell cytotoxicity are both mediated by the same effector cell via different receptors.

In this study, NK and K cell activity of peripheral blood lymphocytes from patients with varying degrees of cervical dysplasia up to carcinoma in situ (CIS) were examined. In addition, the presence of antibody against cytomegalovirus (CMV) in these patients was measured since CMV is one of the candidate agents that may be implicated in the aetiology of carcinoma of the cervix [13].

Materials and Methods

Peripheral blood samples (10 ml) were collected in preservative-free heparin from patients attending the Colposcopy Clinic at Elsie Inglis Maternity Hospital, Edinburgh. The degree of cervical dysplasia observed in frozen sections of biopsies taken from each patient was later confirmed histologically in paraffin sections. Serum samples from 10 ml clotted blood were obtained at the same time and stored at −20 °C.

Effector Cell Isolation

Peripheral blood mononuclear cells were isolated from whole blood within 3 h of removal from the patient by centrifugation through 6 ml lymphopaque separation medium at 1,100 *g* for 20 min at room temperature. Cells harvested from the interface were washed three times in 10 ml Eagle's MEM containing 10% newborn calf serum (Flow Laboratories, Irvine, Scotland), penicillin 100 U/ml and streptomycin 100 µg/ml (TCM). Cells were resuspended in TCM at a final concentration of 1×10^6/ml. These were not further purified.

Target Cells

Target cells for the NK assay were K562 cells, a cell line derived from a patient with chronic myelogenous leukaemia [4] and Chang liver cells. K562 cells were grown in suspension in TCM supplemented with 10% fetal calf serum in addition to newborn calf serum. 1×10^6 K562 cells were resuspended in 100 µl TCM and labelled with 100 µCi [^{51}Cr] sodium chromate (specific activity 350–600 mCi/mg Cr; Amersham) for 60 min at 37 °C. These were then washed three times with 10 ml TCM to remove excess isotope. Cell viability was checked by trypan blue exclusion and cells were resuspended to a final concentration of 1×10^5 viable cells/ml. Chang liver cells were maintained in culture in Nunc plastic flasks in TCM. Cells were removed from the flask with trypsin-versene mixture and 1×10^6 cells labelled with ^{51}Cr as above. Labelled Chang cells were used in both NK and ADCC assays.

Cytotoxicity Assays

NK Assay. 1×10^4 K562 cells were dispensed into 400 µl precipitin tubes (Luckham) in 100 µl volumes. Lymphocyte suspensions (10^5 cells) were added in quadruplicate and the tubes incubated at 37 °C in 5% CO_2 atmosphere for 16–18 h. After incubation all tubes were centrifuged at 400 *g* for 5 min and 100 µl supernatant removed for counting in a gamma counter. Controls with target cells in TCM were included to calculate the spontaneously released isotope. Cells incubated with 2% acetic acid gave the maximum release. Cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{[a - b]}{[c - b]} \times 100,$$

where a = % test release, b = % spontaneous release, c = % maximum release.

ADCC (K Cell Assay). Labelled Chang cells were incubated with either 50 µl normal rabbit serum as a control or 50 µl rabbit anti-Chang cell antiserum for 30 min at 37 °C. Both sera were heat inactivated, then diluted 1:100 for use. Anti-Chang cell antiserum was supplied by Dr. *J. McCormick*, Department of Rheumatology, Northern General Hospital, Edinburgh. Antibody-treated Chang cells were washed twice to remove unabsorbed antibody, resuspended to 1×10^5 viable cells/ml and dispensed in 100-µl volumes. 100 µl effector cell suspension was added (1×10^6/ml) and the tests incubated overnight as above in quadruplicate. Harvesting, counting and calculation for cytotoxicity were performed as above. Controls included target cells plus TCM, target cells plus effector cells and target cells in 2% acetic acid.

Table I. NK and K cell cytotoxicity in patients with cervical abnormalities

Target cell	Percentage mean cytotoxicity ± SD in patients with:			
	normal/mild dysplasia	moderate dysplasia	severe dysplasia	CIS
K562 NK cell assay	36.90 ± 14.31 (27)[1]	39.68 ± 15.54 (35)	38.10 ± 19.50 (21)	31.19 ± 14.14 (22)
Chang NK cell assay	12.98 ± 5.97 (20)	17.81 ± 6.91 (18)	16.83 ± 7.59 (19)	13.47 ± 6.74 (17)
Chang + anti-Chang antibody L cell assay	21.19 ± 8.17 (20)	26.67 ± 7.97 (20)	23.53 ± 6.61 (19)	24.37 ± 6.60 (19)

[1] Number of patients in each group.

Statistics

Significance of the differences between the values obtained for the various groups of patients was calculated by Student's t test.

Anti-Complement Immunofluorescence

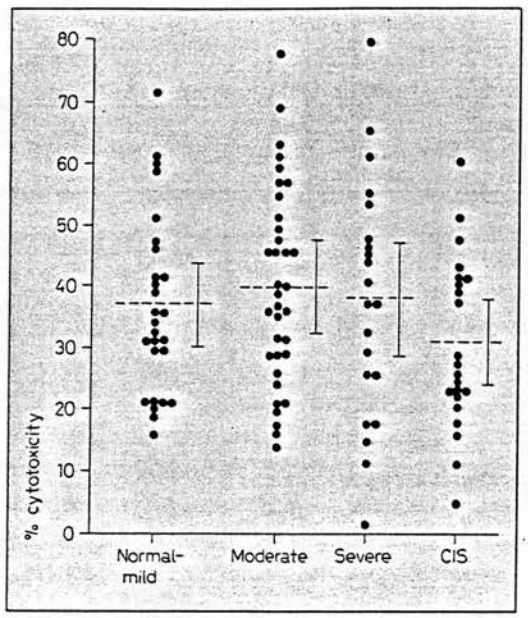
The method of *Kettering* et al. [14] was used in outline to detect antibodies to CMV antigens in the sera of patients. Human embryo fibroblasts were infected with CMV strain AD169 and harvested 3–4 days post-infection. 2×10^4 cells/spot were placed on multi-spot slides (Handley-Essex), allowed to air dry and fixed in cold acetone for 10 min. Sera diluted 1 in 10 were allowed to react for 1 h at 37 °C, human complement (serum sero-negative for CMV) was used at 1 in 4, and anti-human complement C3 labelled with FITC (Hyland Division of Travenol Laboratories) at a dilution of 1 in 30.

Results

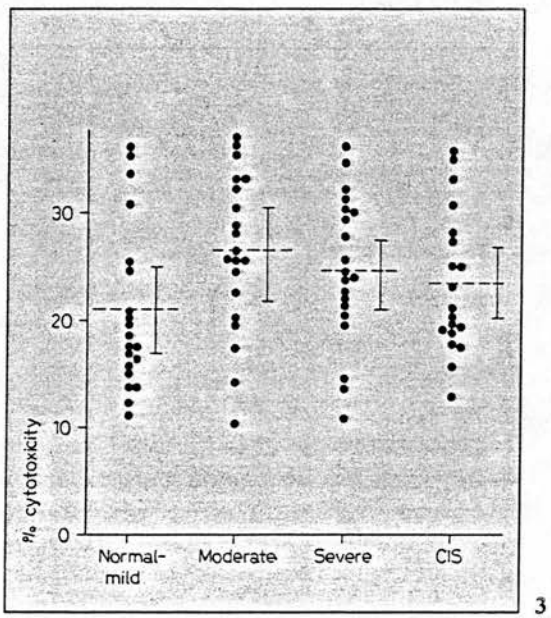
In total, peripheral blood lymphocytes were obtained from 105 patients. Their age and number of pregnancies were noted. They were divided into 4 categories on the basis of the pathology of their cervical biopsies, namely those with normal/mild dysplasia, moderate dysplasia, severe dysplasia and CIS. There were roughly equal numbers of patients in each group. Initially NK cell activity was measured with K562 cells as targets but this was expanded to include Chang cells as NK targets and also K cell activity in studies with an ADCC test on Chang cells as targets.

Table I shows the mean ± SD of NK and K cell cytotoxicity values of lymphocytes from the four grops of patients studied. While there is a reduction in the mean NK value observed within the group of patients with CIS (31.19 ± 14.14) when compared with the value obtained for those with normal/mild dysplasia (36.90 ± 14.31), moderate dysplasia (39.68 ± 15.54) and severe dysplasia (38.10 ± 19.50), the difference between the means was not significant ($p < 0.1$). Figure 1 shows the individual results obtained for NK cytotoxicity where there was wide variation in NK activity within the various patient groups, most obviously in those patients with severe dysplasia whose NK values ranged from 0.69 to 79.54%.

Figure 2 shows the individual results obtained for NK cytotoxicity towards Chang cells. These values reflected the decreased sensitivity of Chang cells to NK lysis when compared with K562 cells. The mean values for Chang cell NK activity shown in table I demonstrate no significant differences between the patient groups.



1



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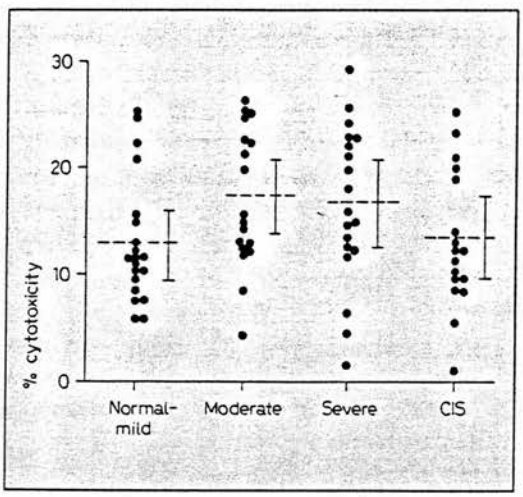

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Fig. 1. Individual results of NK cell cytotoxicity towards K562 target cells ± SD.

Fig. 2. Individual results of NK cell cytotoxicity towards Chang target cells ± SD.

Fig. 3. Individual results of ADCC towards Chang target cells coated with 1:100 anti-Chang cell antibody.

Table I also gives results from 78 patients whose lymphocytes were assayed for K cell cytotoxicity in the ADCC test. No significant differences were found between the patient groups. The individual results are given in figure 3.

Effect of Age and Number of Pregnancies on NK Activity

No significant differences were observed within the various groups of patients when age and number of pregnancies were considered (table II).

Table II. Age and number of pregnancies in patients with cervical abnormalities

	Patient group			
	normal/mild dysplasia	moderate dysplasia	severe dysplasia	CIS
Age ± SD	32.54 ± 6.17	29.00 ± 5.51	31.70 ± 5.42	31.63 ± 4.39
Pregnancies ± SD	2.46 ± 2.06	1.74 ± 1.77	2.04 ± 1.68	2.28 ± 1.67

CMV Antibody Data

A total of 87 sera were examined by ACIF for antibodies to CMV. The proportion of sera sero-positive for CMV in each group was as follows: normal/mild 14/27; moderate 12/23; severe 16/22 and CIS 11/15. The difference between any group when compared with CIS failed to reach statistical significance by Chi-square test.

When the NK values obtained with K562 cells from sero-positive patients were compared with those sero-negative for CMV, there was no significant difference between the means (sero-positive 38.26 ± 16.14%; sero-negative 37.80 ± 15.28%).

Discussion

No evidence was found in this study to indicate any decrease in NK activity in patients with cervical abnormalities up to CIS. These findings are in agreement with those of several groups who have examined patients with localized tumours of many types [15, 16]. NK activity, however, does appear to be decreased in cancer patients with metastasis, untreated lymphocytic leukaemia and melanomas [9–11, 17]. Patients with carcinoma of the cervix have been studied previously [12], but only in advanced stages of the disease when it had progressed from CIS to invasive cancer. Although the number of patients in each group in that study was not large, progressive decrease of NK activity was demonstrated as the disease spread, with those in relapse having the lowest NK activity of all. It therefore appears that, if NK cells possess a role in preventing the disease from firstly becoming established, and secondly from progressing, it is an inefficient system which loses much of its effectiveness when the tumour grows. On the other hand, lymphocyte infiltration is common in tumours of patients with CIS. It may, therefore, be argued that the presence of NK cells or cytotoxic T lymphocytes maintains the CIS status and prevents spread. It is known that patients may remain at CIS level for many years before progressing to invasive carcinoma so it is apparent that some forces are acting upon the tumour to prevent its spread.

In the present study, great variation in the NK activity within all the groups of patients was demonstrated so that it was not possible on the strength of this function alone to identify those patients with mild or moderate dysplasia who might be likely to progress to CIS or invasive carcinoma. Additionally, the finding that NK cells are active in vitro

against sensitive targets such as K562 cells does not necessarily indicate that those same NK cells could kill autologous tumour cells in vitro.

CMV has been isolated from cells cultured from cervical tumours [13] and has also been associated with human malignancies, in particular, Kaposi's sarcoma [18] and carcinoma of the prostate [19]. CMV has also been shown to be capable of transforming normal cells into cells able to produce tumours when injected into susceptible animals [20]. Since NK cells are particularly active against virus-infected targets, the CMV antibody status of the patients in the study was examined for any correlation with NK activity. It is assumed that CMV antibody would be increased in the presence of a 'chronic viral' infection. No such correlation was seen either when all patients, regardless of degree of dysplasia, were divided into sero-positive or sero-negative groups or when, for example, CIS patients sero-positive for CMV were compared with those sero-negative. Although the proportion of patients with CIS who were sero-positive for CMV was greater than the proportions of sero-positive patients with dysplasia, the difference was not statistically significant. A larger study had previously shown no difference in the anti-CMV antibody status of patients with CIS when compared with other dysplasias [21], and our results confirm this.

Acknowledgements

We wish to thank Dr. *G. Smart* and Dr. *J. Livingstone*, Mrs. *M. Murray* and the Staff of the Colposcopy Clinic, Elsie Inglis Hospital, Edinburgh, for their generous help in supplying the clinical specimens. This study was supported by a grant from the Cancer Research Campaign.

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Received: January 13, 1984
Accepted: May 3, 1984

W. Neill,
Department of Bacteriology,
University of Edinburgh Medical School,
Edinburgh EH8 9AG (UK)

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Elution of cytomegalovirus antibodies from adenocarcinoma of the colon

ANN GRAIL AND MARY NORVAL

Reprinted from Gut
Volume 26, No. 10. pages 1053-1058, October 1985.

LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, WC1H 9JR

Gut, 1985, **26**, 1053–1058

Elution of cytomegalovirus antibodies from adenocarcinoma of the colon

ANN GRAIL AND MARY NORVAL

From the Department of Bacteriology, University of Edinburgh Medical School, Edinburgh

SUMMARY Eluates were prepared by high salt extraction from normal colonic mucosa and adenocarcinomatous tissue from 28 patients, eight more from unmatched colonic tissue and five from patients with other gastrointestinal disease. Immunoglobulins were detected by ELISA: IgG was present in 24% eluates from normal colon and 21% from carcinomas; IgA in 55% eluates from normal colon and 39% from carcinomas; IgM in 55% from normal colon and 37% from carcinomas. Cytomegalovirus-specific antibody was found in 15% eluates from normal colon and in 18% carcinomas. Out of the 28 matched specimens, cytomegalovirus-specific IgG was detected in one normal and four tumour eluates, specific IgA in two normal and four tumour eluates, and specific IgM in two normal and two tumour eluates. In two instances cytomegalovirus-specific antibody was present in the eluates prepared from the normal and tumour tissue of the same patient. Of those eluates which contained cytomegalovirus-specific antibodies by ELISA, two were positive by anti-complement immunofluorescence of human embryo fibroblasts infected with cytomegalovirus strain AD-169. It seems possible, therefore, that cytomegalovirus antigens on colonic cells may be masked by complexing with anti-cytomegalovirus antibodies, and may not therefore be detected by techniques such as immunofluorescence.

Most cytomegalovirus infections of man are asymptomatic although they can occasionally result in congenital defects, infectious mononucleosis, interstitial pneumonia, hepatitis, and other severe diseases.[1] After the primary infection, the virus persists in the body by unknown mechanisms and in unknown sites, although peripheral blood leucocytes seem to represent one cell population from which virus can be recovered on occasion.

There has been suggestive evidence that cytomegalovirus may be involved in the pathogenesis of certain types of cancer, particularly Kaposi's sarcoma,[2] carcinoma of the cervix[3] and prostate,[4] and adenocarcinoma of the colon. Thus in 1978 Huang and Roche[5] detected cytomegalovirus-DNA by hybridisation in four of seven patients with carcinoma of the colon, while Hashiro and coworkers[6] isolated cytomegalovirus from cultured tumour fragments in three of eight cases. More recently, however, Brichacek and others[7] failed to detect cytomegalovirus DNA, using DNA-DNA hybridisation with an estimated sensitivity of 0·3 genome equivalents per cell, in biopsy specimens from seven patients with colonic carcinoma. In addition we failed to confirm an involvement of cytomegalovirus using hybridisation, immunofluorescence to detect cytomegalovirus-specific antigens, and by culture of biopsy specimens.[8] Roche *et al*[9] have looked again at the role of cytomegalovirus in gastrointestinal disease and concluded that the virus was detectable by DNA-reassociation kinetics in a proportion of colonic cells, including those derived from both malignant and non-malignant tissue. It was suggested that cytomegalovirus was present in masked or latent form, and no biopsy specimens were positive for viral antigens by immunofluorescence. It is possible that such antigens on the surface of tumour cells could be blocked by complexing with antibodies as has been suggested recently.[10] Indeed just such a situation has been described for *Herpes simplex* virus-specific antibodies in squamous cell carcinoma of the uterine cervix in the absence of apparent infection of the tumour cells by the virus.[11]

Thus the present study was undertaken to identify

Address for correspondence: Dr A Grail, Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.

Received for publication 22 November 1984

bound immunoglobulins in normal and adenocarcinomatous colonic mucosa, and to identify whether any was cytomegalovirus specific.

Methods

CLINICAL SPECIMENS

Surgically resected specimens of colon were obtained from the Royal Infirmary, Edinburgh. Diagnosis and Duke's stage, where appropriate, was based on routine pathological examination. Thirty eight specimens of tumour and 33 of normal colonic mucosa were collected which included 28 matched specimens of tumour and normal colon taken from a region of the gut uninvolved by tumour. The remaining specimens were six unmatched carcinoma of the colon, two normal colon mucosa, one Crohn's disease, one benign adenoma with dysplasia, two carcinoma of the caecum and one gastric carcinoma leading into the bowel. All tissues were stored at −70°C until elution was performed.

ELUTION OF ANTIBODIES

A modification of the method of Boyd and James[12] was used. Portions of tissue (0·5–5 g) were finely chopped and washed three times in 0·01M phosphate buffered saline, pH 7·2 (PBS). Solid material was further disaggregated in nine volumes of an enzyme mixture containing 10 mg collagenase B (Sigma) and 4 mg DNase (BDH) in 100 ml PBS for 20 min at 37°C with gentle stirring. The resulting cell suspension was removed onto ice and the procedure repeated twice on any solid material. Cell suspensions were washed five times in PBS and resuspended in 5 ml 15% NaCl (pH 7·4) before being incubated for two hours at 37°C. The eluate was obtained by centrifugation at 10 000 *g* for 10 minutes. The sediment was re-extracted with 15% NaCl as before. Resultant eluates were pooled and dialysed against 500 ml distilled water for 24 hours with two changes of water. After centrifugation for one hour at 36 000 *g*, the supernatant was concentrated 10 times with polyethylene glycol and finally centrifuged for 30 min at 5000 *g*. Eluates were stored frozen at −20°C. Protein estimation was by the method of Lowry with bovine serum albumin as standard.

DETECTION OF IMMUNOGLOBULINS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The procedure was a modification of the method described by Bidwell, Bartlett, and Voller.[13] The assay was done on polystyrene, flat bottomed micro titre plates (Sterilin). Wells were saturated with 50 μl of 175 μg/ml goat anti-human polyvalent immunoglobulins (Sigma) diluted in 0·05M carbonate buffer, pH 9·6, for four hours at 37°C, and then overnight at 4°C. Plates were washed three times with 0·05% Tween 20 containing 0·9% NaCl. Fifty microlitres of undiluted eluate was added and the plates incubated at room temperature for six to eight hours. After washing again, 50 μl of goat anti-human IgA, IgG, or IgM conjugated with alkaline phosphatase (Sigma, diluted in 0·05M PBS containing 0·05% Tween 20 according to the manufacturer's instructions) was added, and the plates incubated at room temperature. Absorbance at 405 nm was measured with a multiscan (Titertek, Flow). In each experiment positive controls were myeloma sera specific for IgA, IgG, or IgM; negative control values were obtained from the same assay system but without the eluates (these are shown in the Table). Values greater than the mean+standard deviation of the control values were considered positive. Chi-square tests were used to determine any significant differences in the detection of immunoglobulins in normal and tumour eluates and with respect to the stage of the tumour. Wilcoxon's rank sum tests were used to compare the concentrations of each immunoglobulin present in the 28 normal and tumour eluates, and to compare immunoglobulin concentrations with respect to the stage of the tumour.

Detection of cytomegalovirus-specific immunoglobulins was done in a similar manner but with cytomegalovirus-infected cell antigen or uninfected cell antigen (see below) as the initial coating layer. Antigens were diluted from 400 to 1000 times in 0·05M carbonate buffer pH 9·6 for ELISA. Each eluate was tested against control antigen and cytomegalovirus antigen because there may be antibodies in the eluates which cross react with antigens from human embryonic fibroblasts. The mean and standard deviation of eluates tested against control human embryonic fibroblast antigen allowed calculation of a positive reaction to cytomegalovirus antigen as is shown in the legend to the Table. Chi-square tests were used to determine any significant differences in the detection of cytomegalovirus-specific immunoglobulins in normal and tumour eluates, and with respect to tumour stage. There were too few eluates containing cytomegalovirus-specific immunoglobulin to compare the concentrations of immunoglobulins in normal and tumour eluates or with respect to tumour stage.

CYTOMEGALOVIRUS CELL ANTIGEN PREPARATION

Human embryonic fibroblasts at pass 3 to 10 were grown in modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 200 μg/ml streptomycin. When confluent some cells were infected with cytomegalovirus, strain AD-169,

Table *Non-specific and cytomegalovirus-specific immunoglobulins as measured by ELISA (Optical Density) in eluates from colonic adenocarcinoma (1–34), normal colonic mucosa (1–28, 35, 36), Crohn's disease (37), benign adenoma with dysplasia (38), carcinoma of the caecum (39, 40) and gastric carcinoma (41). Cytomegalovirus-specific anti-complement immunofluorescence of eluates is also shown (+ positive; − negative) and their protein concentration (mg/g wet weight tissue).*

		Eluates from carcinoma of colon								*Eluates from normal mucosa*							
		Protein	*Non-specific*			*CMV-specific*				*Protein*	*Non-specific*			*CMV-specific*			
Patient no	*Duke's stage*	*mg/g*	*IgG*	*IgA*	*IgM*	*IgG*	*IgA*	*IgM*	*ACIF*	*mg/g*	*IgG*	*IgA*	*IgM*	*IgG*	*IgA*	*IgM*	*ACIF*
1	B	0·29	0·84	1·17	≥2	1·8	2·0	1·4	−	0·78	0·89	1.23	≥2	0·35	0·17	0·11	−
2	C	0·64	0·72	0·74	≥2	≥2	0·37	0·4	−	0·2	1·03	0·85	≥2	0·23	1·6	0·41	−
3	C	0·4	0·97	0·89	1·73	≥2	1·41	1·82	+	0·26	0·98	0·25	1·85	≥2	0·46	0·95	+
4	C	0·1	0·74	0·77	1·48	0·36	0·17	0·12	−	0·17	0·32	0·44	0·46	0·37	0·29	0·38	−
5	C	0·09	0·37	0·42	1·56	0·57	0·43	0·35	−	0·58	0·25	0·43	0·27	0·45	0·36	0·28	−
6	B	0·34	0·33	0·78	1·22	0·49	0·43	0·29	−	0·51	0·43	0·46	0·43	0·73	0·21	0·51	−
7	C	0·42	0·32	1·81	0·73	0·71	0·26	0·37	−	0·67	0·39	0·41	1·45	0·21	0·37	0·39	−
8	A	0·31	0·72	0·36	1·35	0·43	0·31	0·41	−	0·54	0·42	0·33	≥2	0·46	0·27	0·37	−
9	B	0·26	0·77	0·79	0·34	0·35	0·25	0·29	−	0·35	0·38	0·34	0·37	0·44	0·49	0·18	−
10	C	0·35	0·37	0·9	1·76	0·68	1·4	0·47	−	0·4	0·35	0·29	1·65	0·36	0·42	0·26	−
11	A	0·42	1·14	0·41	0·32	0·89	0·4	0·37	−	0·76	0·88	0·86	1·34	0·41	0·35	0·24	−
12	C	0·33	0·34	0·92	0·35	0·46	0·39	0·27	−	1·2	0·34	1·18	0·79	0·26	0·45	0·41	−
13	C	0·42	0·42	1·49	0·27	0·27	0·71	0·19	−	1·2	0·27	0·79	1·36	0·14	0·36	0·13	−
14	C	0·2	0·38	0·46	1·27	0·48	0·34	0·35	−	0·52	0·26	0·79	0·37	0·69	0·23	0·18	−
15	C	0·38	0·44	0·89	0·36	0·2	0·89	0·27	−	0·45	0·24	1·52	0·78	0·38	0·19	0·46	−
16	C	0·07	0·36	0·35	0·29	0·44	0·28	0·49	−	0·7	0·44	0·43	0·47	0·53	0·62	0·26	−
17	B	0·14	0·35	0·45	0·27	0·31	0·57	0·26	−	1·1	0·4	0·98	1·23	0·43	0·56	0·18	−
18	A	0·06	0·27	0·36	0·47	0·24	0·23	0·6	−	0·4	0·45	0·87	1·39	0·46	0·32	0·29	−
19	C	0·39	0·37	0·44	0·46	0·41	0·16	0·15	−	0·2	0·39	0·47	0·46	0·73	0·77	0·61	−
20	A	0·22	0·46	0·35	0·38	0·47	0·28	0·18	−	0·31	0·42	0·87	1·83	0·49	0·42	0·25	−
21	B	0·18	0·36	0·4	0·43	0·28	0·15	0·47	−	0·29	0·38	1·08	1·5	0·41	0·37	0·28	−
22	B	0·08	0·28	0·42	0·38	0·23	0·14	0·39	−	0·29	0·44	0·85	1·43	0·34	0·45	0·21	−
23	B	0·18	0·4	0·37	0·42	0·41	0·17	0·12	−	0·39	1·06	0·8	0·38	0·16	0·36	0·44	−
24	B	0·1	0·44	0·31	0·49	0·21	0·12	0·48	−	0·27	0·39	0·39	0·37	0·25	0·29	0·3	−
25	C	0·15	0·42	0·38	0·45	0·29	0·37	0·22	−	0·05	0·45	0·37	0·39	0·28	0·34	0·26	−
26	C	0·34	0·31	0·42	0·5	0·64	0·28	0·33	−	0·63	0·43	1·01	0·41	0·23	0·29	0·37	−
27	B	0·03	0·44	0·45	0·35	0·56	0·72	0·18	−	1·09	0·38	0·87	≥2	0·4	1·42	1·17	−
28	B	0·3	0·32	0·46	0·36	0·29	0·42	0·26	−	0·42	0·29	0·25	0·49	0·22	0·29	0·28	−
29	B	0·26	0·26	0·41	0·42	0·15	0·19	0·19	−								
30	C	1·02	0·32	0·36	0·41	0·24	0·16	0·39	−								
31	B	0·12	0·45	0·44	0·22	0·18	0·12	0·56	−								
32	C	0·36	0·35	0·94	1·42	0·47	0·43	0·36	−								
33	B	0·32	0·46	0·36	1·71	0·49	0·49	0·15	−								
34	B	0·38	0·47	0·29	0·36	0·31	0·17	0·17	−								
35										1·22	0·97	0·91	≥2	0·19	0·41	0·42	−
36										0·31	0·89	0·87	0·37	1·04	0·96	0·37	−
37										0·18	0·35	0·34	0·29	0·56	0·24	0·22	−
38		0·5	0·24	0·87	0·98	0·14	0·42	0·37	−	0·63	1·08	0·86	0·32	0·24	0·27	0·15	−
39		0·27	0·25	0·36	0·44	0·26	0·41	0·22	−								
40		0·09	0·93	0·87	1·55	1·48	0·39	0·26	−								
41		0·23	0·34	0·86	0·49	0·21	0·13	0·24	−	1·6	0·32	0·42	0·95	0·18	0·11	0·96	−

Control values for non-specific antibodies: IgG mean 0·42 standard deviation 0·06
IgA mean 0·39 standard deviation 0·09
IgM mean 0·43 standard deviation 0·07
Control values for CMV-specific antibodies: IgG mean 0·52 standard deviation 0·22
IgA mean 0·54 standard deviation 0·25
IgM mean 0·44 standard deviation 0·18
Values greater than the mean + standard deviation were considered positive; these values are underlined.

at 1–2 plaque-forming units per cell. Infected and uninfected cultures were maintained in medium containing 2% fetal calf serum at 37°C until advanced cytopathic effects were observed in the infected culture. The monolayers were washed with cold PBS and harvested using glass beads. Cells (5×10^6) were washed three times in PBS and resuspended in 1 ml glycine buffer (5 parts 0·1M

glycine plus 3 parts 0·1M NaOH, prepared on day of use). They were sonicated for two minutes at 9 kc/s and stored at −70°C until required.

CYTOMEGALOVIRUS-SPECIFIC ANTI-COMPLEMENT IMMUNOFLUORESCENCE TEST

Monolayers of human embryonic fibroblast cells grown on cover slips were infected with cytomegalovirus, strain AD-169, and 48 h later were fixed in cold acetone for 10 minutes. Anticomplement immunofluorescence (ACIF) was carried out by the method of Hart *et al.*[8]

Results

Normal colonic mucosa and slices of adenocarcinomas were disaggregated with collagenase and DNase, and 71 eluates prepared from the resultant single cell suspension after treatment with 15% NaCl. Protein concentrations of eluates ranged from 0·07–1 mg/ml, mean value 0·48 mg/ml; for carcinoma eluates a mean value of 0·28±0·19 (standard deviation) mg/g of tissue, and for normal eluates 0·57±0·37 (standard deviation) mg/g of tissue were found.

Using ELISA, out of 38 specimens of tumour, IgG was detected in eight eluates, IgA in 15 and IgM in 14 (Table). Out of 33 specimens of normal colon, IgG was detected in eight eluates, IgA in 18 and IgM in 18.

These results indicate that there is no significant difference in the detection of IgG (24%), IgA (55%) and IgM (55%) in normal eluates compared with tumour eluates (21% contained IgG, 39% contained IgA and 37% contained IgM ($0{\cdot}1<p<0{\cdot}5$)). There were no significant differences in the immunoglobulin concentrations of normal and tumour eluates. When the 28 matched pairs of normal and tumour eluates from the same patient were compared (shown in the Table, patients 1–28), five normal and seven tumour eluates contained IgG, 15 normal and 11 tumour eluates contained IgA, and 15 normal and 10 tumour eluates contained IgM. These results also indicate that there is no significant difference in the detection of IgG, IgA and IgM in the 28 matched eluates. In three pairs of specimens the same immunoglobulin types were present in both the normal and tumour eluates, and in five matched cases no immunoglobulins were detected in eluates from either normal or tumour tissues.

Cytomegalovirus-specific antibody as measured by ELISA was found in seven of 38 tumour eluates and five of 33 normal colon eluates (also shown in the Table). Comparing the 28 matched specimens, cytomegalovirus-specific IgG was detected in one normal and four tumour eluates, cytomegalovirus-specific IgA in two normal and four tumour eluates and cytomegalovirus-specific IgM in two normal and two tumour eluates. These results show no significant difference in the presence of cytomegalovirus-specific IgG, IgA and IgM in tumour eluates as compared with normal eluates ($0{\cdot}1<p<0{\cdot}5$). In two instances, cytomegalovirus-specific immunoglobulins were present in the eluates from both the tumour and normal tissue of the same patient, although of the same broad immunoglobulin class in only one.

The patients from whom the specimens were obtained had colorectal adenocarcinoma for the most part, and were grouped histologically according to the Duke's system of classification; a few had other bowel disorders (Table). Four out of 34 patients with colonic carcinoma had Duke's stage A, 14 of 34 had stage B, and 16 of 34 had stage C. Of the patients with stage A disease, two of four carcinoma eluates contained detectable immunoglobulin; this was IgG in both cases and in one case IgA was also present. Four of 14 carcinoma eluates from patients with Duke's stage B were positive for immunoglobulin; IgG was present in two eluates, IgA in three eluates and IgM in three eluates. Eleven of 16 eluates from patients with stage C disease contained immunoglobulin; IgG was present in three eluates, IgA in nine eluates and IgM in eight eluates. These results show no significant differences in the detection of IgG (19%) ($p>0{\cdot}5$), IgA (56%) ($0{\cdot}05<p<0{\cdot}1$) and IgM (50%) ($0{\cdot}1<p<0{\cdot}5$) in eluates from patients with stage C disease compared with stage B disease (IgG was detected in 14% and IgA and IgM were detected in 21% eluates). No significant differences in the immunoglobulin concentrations were found with respect to tumour stage.

Cytomegalovirus-specific immunoglobulin was detected in one patient with Duke's stage A, one patient with stage B and four patients with stage C. There is no significant increase in the detection of cytomegalovirus-immunoglobulin in eluates from patients with stage C tumour (25%) compared to stage B tumour (7%) ($0{\cdot}1<p<0{\cdot}5$).

Anti-complement immunofluorescence was performed using human embryonic fibroblast cells infected with cytomegalovirus, strain AD-169, and uninfected human embryonic fibroblasts as controls. Eluates prepared from both normal and tumour tissue of patient number three showed positive immunofluorescence.

Although the eluates from the colonic tissues contained varied amounts of protein, the nature of these, apart from IgA, IgG, IgM and cytomegalovirus-specific immunoglobulins was not determined.

Discussion

Elution of bound immunoglobulin from normal colonic mucosa and colorectal carcinoma was achieved by high salt extraction. There were no significant differences in the detection of IgG, IgA and IgM in normal and carcinoma eluates: IgG was present in 24% of normal mucosa eluates and 21% of carcinoma eluates ($p>0{\cdot}5$): IgA was present in 55% of normal eluates and 39% of carcinoma eluates ($0{\cdot}1<p<0{\cdot}5$): IgM was present in 55% of normal eluates and 37% of carcinoma eluates ($0{\cdot}1<p<0{\cdot}5$). Detection of IgG, IgA, IgM and cytomegalovirus-specific immunoglobulin was not significantly different in eluates from patients with Duke's stage C compared with eluates from stage B disease.

MacSween and Eastwood[14] found significantly higher concentrations of IgG in eluates of colonic carcinoma with regional metastasis than in eluates of localised tumours. This difference was not apparent in eluates of corresponding normal tissue, and they suggested that the increase in IgG was associated with the metastatic state. Also it has been proposed that patients with colorectal carcinoma who initially have higher serum levels of IgG and perhaps of IgA may have a longer relapse free survival.[15] More recently, all tumours have been reported to contain IgG and IgA, whereas only 40% contained measurable amounts of IgM.[16] These differences in immunoglobulin detection from the present study may reflect use of different or larger specimens from patients or differences in the preparation of eluates. In addition the ELISA used here employed polyvalent antiserum as the first layer which may have resulted in competition for immunoglobulin binding sites and therefore reduced detection of individual immunoglobulins. Jones *et al*[16] found less IgA, IgM and IgG in eluates from colorectal carcinoma than in normal colonic mucosa. Also there was no association between immunoglobulin and the amount of tumour tissue, necrosis, mucin production and staging, although it was suggested that tumour associated IgM accompanied an unfavourable prognosis. In the present study detection of IgG, IgA, IgM and cytomegalovirus specific immunoglobulin was not significantly different in stage C disease compared with stage B. Further interpretation of an association between immunoglobulins and staging is prevented by the small number of eluates from patients with stage A tumour.

There were no significant differences in the detection of cytomegalovirus specific IgG, IgA and IgM in the 28 matched samples. The presence of cytomegalovirus specific immunoglobulin in normal and tumour eluates suggests that some cells in the colonic mucosa may be expressing cytomegalovirus specific antigens on their surfaces to which the antibodies are binding. It also implies that such antigens will be blocked and thus escape detection by such techniques as immunofluorescence. It is of interest in this context that Roche *et al*,[9] while showing cytomegalovirus DNA in various colonic diseases by hybridisation, were unable to show cytomegalovirus antigens by immunofluorescence. Only two eluates in our series were positive for cytomegalovirus antibodies using anticomplement immunofluorescence of AD-169 infected fibroblasts which almost certainly reflects the greater sensitivity of the ELISA method over anticomplement immunofluorescence.

The presence of cytomegalovirus immunoglobulins in eluates from both normal and tumour material suggests that cytomegalovirus infection is not associated specifically with colonic adenocarcinomas. Furthermore, in two cases, such antibodies were detected in eluates from tumour and normal colonic mucosa of the same patient. A distinction was not made between antibody eluted from infiltrating lymphocytes or from epithelial cells in the adenocarcinomatous tissue and the normal colonic mucosa. Immunofluorescence on sections of colon may resolve this, although recent evidence from virus isolation and nucleic acid hybridisation would indicate that the presence of the virus, at least, is not solely because of infiltrating mononuclear cells infected with cytomegalovirus.[9]

It is known that, after a primary infection, cytomegalovirus persists in a latent form within man.[1] As a number of studies has shown the presence of cytomegalovirus in the gastrointestinal tract either in normal patients, those with chronic diseases such as ulcerative colitis,[17] or in colorectal adenocarcinoma,[5] the gastrointestinal tract has been suggested as a possible site of latency of cytomegalovirus.[9] The present study, thus, would also indicate that cytomegalovirus is found in normal colonic and adenocarcinoma cells by the detection of cytomegalovirus specific immunoglobulins in tissue eluates from some patients. It cannot be discounted, however, that some proportion of these cytomegalovirus antibodies may be sticking non-specifically. Further work is necessary to establish whether cytomegalovirus is associated with transformation of normal cells to the malignant state in the colon, whether it is the initiating factor or a cofactor, or whether it is not involved at all in this process.

We wish to acknowledge the very kind help of Mr I MacLeod of the Royal Infirmary, Edinburgh, and Dr H Gilmour of the Department of Pathology,

University of Edinburgh. This work was supported by the Cancer Research Campaign.

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0378–7346/85/0202–0096$2.75/0

Cell-Mediated Immune Responses to Cytomegalovirus in Patients with Dysplasia of the Uterine Cervix

W.A. Neill, M. Norval

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, UK

Key Words. Immunity, cell-mediated · Cytomegalovirus · Lymphocyte transformation · Dysplasia of uterine cervix · Enzyme-linked immunosorbent assay

Abstract. Peripheral blood lymphocytes from 172 women with various degrees of cervical dysplasia were assayed for responsiveness to cytomegalovirus-induced early and late antigens using lymphocyte stimulation tests. No evidence was found to suggest that any group of patients was specifically sensitized to either of the antigens tested. Neither was there any difference between the patient groups in respect of mitogen-induced stimulation. The concept that cytomegalovirus might be involved in cervical cell transformation leading to carcinoma has therefore not been strengthened as a result of this study.

Introduction

The cell-mediated immune (CMI) response is of extreme importance in recovery from herpesvirus infections. In particular, patients receiving cardiac [1], renal [2] or bone marrow [3] transplants often experience either reactivation of endogenous cytomegalovirus (CMV) or primary CMV infection leading to high morbidity and mortality. Such patients are generally immunosuppressed to minimize host versus graft responses, and CMI responses to CMV are also compromised, although humoral antibody status is unaffected.

CMV has recently been considered as an agent with possible involvement in various human neoplasms [4–6] including carcinoma of the uterine cervix. Serological studies on patients with grades of cervical dysplasia up to carcinoma in situ (CIS) have been equivocal with regard to CMV antibody status [7–10], while CMI responses in such patients have not yet been examined.

The lymphocyte stimulation (LS) test, an in vitro parameter of CMI, has been pre-

viously used to demonstrate specific lymphocyte responsiveness to CMV in several groups of patients including transplant recipients [11] and individuals with acquired immune deficiency syndrome (AIDS) [12]. The present study examined the response of peripheral blood lymphocytes (PBL) to CMV in patients with various grades of cervical dysplasia up to CIS. Lymphocyte responsiveness to CMV was then correlated with the presence of CMV antibody in serum, measured by an enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Subjects and Specimens. The study population comprised 172 women attending the Lothian Area Colposcopy Clinic, Edingburgh. Diagnosis of grade of dysplasia was made on frozen sections of cervical biopsies and later confirmed on paraffin sections. Ten ml peripheral blood was collected from each patient into 100 units of preservative-free heparin (Sigma). A further sample was taken at the same time to provide serum for the ELISA test. The specimens arrived at the laboratory within 2 h of collection.

Preparation of PBL. PBL were prepared as previously described [13] and resuspended at 1×10^6 cells/ml in RPMI 1640 supplemented with 15% autologous plasma, 100 units/ml penicillin and 100 µg/ml streptomycin.

Preparation of CMV Antigens. Confluent monolayers of human embryo fibroblasts (HEF) were infected with CMV strain AD169 and harvested with glycine buffer when an extensive cytopathic effect was seen [14]. This preparation was referred to as CMVLA. For the production of the CMV early antigen (CMVEA) preparation, HEF cells were harvested 24–48 h after infection. Control antigen (HEF) was prepared in the same way from uninfected cells. Antigens were stored in aliquots at −70 °C until used.

Preparation of Mitogens. Three mitogens were employed in the study: these were phytohaemagglutinin (PHA, Wellcome), pokeweed mitogen (PWM, Gibco) and concanavalin A (Con A, Sigma). Dilutions of these were set up in RPMI 1640 and dose-response curves set up as decribed below to find the optimum concentration for routine use.

The LS Test. PBL at 1×10^6/ml were dispensed in 200-µl volumes into round-bottomed 96-well microtest plates (Nunc) in quadruplicate. Antigens and mitogens were added at varying concentrations and the plates incubated at 37 °C in 5% CO_2 and 95% humidity. On the day prior to harvesting, 0.5 µCi ^{3}H-methyl thymidine (Amersham) was added to each well. Cells were harvested 24 h later onto fibre glass strips in an automatic sample harvester (Ilacon). After drying, individual discs were counted in PPO/POPOP toluene-based liquid scintillator for 1 min each. LS was expressed as the stimulation index (SI). SI = Mean counts per minute (cpm) of PBL with CMVLA or CMVEA + mean cpm of PBL with HEF antigen. In tests with mitogens, SI = mean cpm of PBL with mitogen + mean cpm of PBL alone. A SI value of 2.0 or more was accepted as positive stimulation in accordance with other groups [15].

Elisa. The method used was based on that described by *Booth* et al. [14]. Briefly, 96-well immunoplates (Nunc) were coated overnight at 4 °C with CMVLA or HEF glycine-extracted antigen at optimal concentration (1:600) as assessed by chessboard titration. All test sera were assayed at 1:100 dilution in buffer containing 1% bovine serum albumin. Alkaline phosphatase-labelled anti-human IgG (Miles-Yeda) was used at 1:200 dilution and phosphatase substrate (Sigma) at 1 mg/ml. Absorbance of samples was read in a Dynatech Microelisa Autoreader at 405 nm. Mean ± standard deviation (SD) absorbance of each serum was calculated and any sample giving a value greater than that obtained for a negative control serum with CMV antigen plus 3 × SD was considered positive. In addition, the absorbance value obtained for each serum sample was used as a measure of antibody titre in calculations to correlate ELISA with SI values.

Results

Initial experiments were performed to establish the concentrations of CMVLA and mitogens which gave maximum lymphocyte stimulation in 6 normal healthy women, 5 of whom were seropositive (> 1:4) by comple-

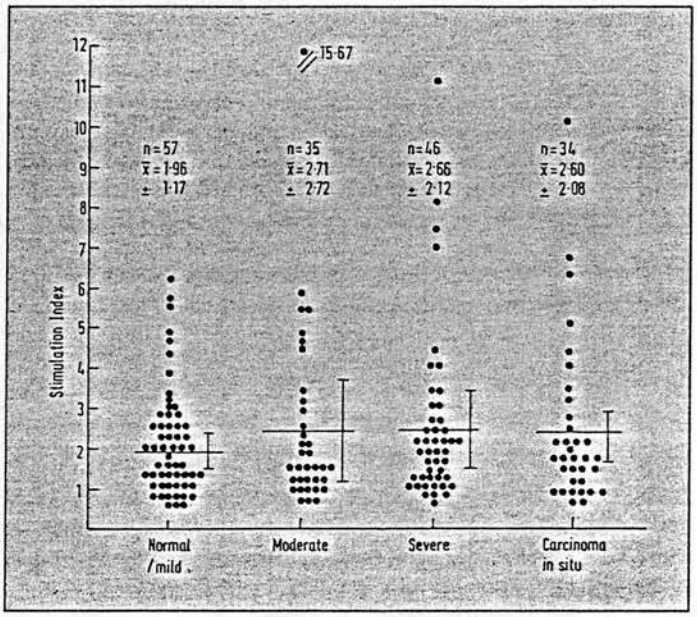

Fig. 1. Stimulation index (mean ± SD) of PBL from groups of patients with grades of cervical dysplasia towards CMVLA.

ment fixation test (CFT). PBL from the seronegative donor failed to reach a SI of 2.0 when incubated for 5–7 days with CMVLA at concentrations ranging from 1:20 to 1:640. On the other hand, the mean (± SD) SI of the seropositive donors was 3.92 ± 2.12 (range 2.77–7.71) at 1:80, 4.53 ± 1.99 (range 2.62–7.31) at 1:160 and 4.53 ± 2.36 (range 1.93–8.34) at 1:320 concentration of CMVLA. Antigen at 1:20 and 1:40 was lymphotoxic resulting in a SI of less than 1.0. The optimal time for harvesting was after 6 days of incubation. Since the concentration of CMVLA which resulted in maximum stimulation varied among the seropositive donors, assays on patients' PBL were set up at all three concentrations. The concentration of mitogens resulting in maximum stimulation of the test PBL was PHA 1:160 (6 of 6 donors), PWM 1:40 (6 of 6) and Con A 0.3 μg/ml (5 of 6). These cultures were also harvested after 6 days of incubation.

PBL were tested from a total of 172 patients between the ages of 20 and 41. These were divided into 57 patients with no cervical abnormality, cervicitis or mild dysplasia, 35 with moderate dysplasia, 46 with severe dysplasia and 34 with CIS. Whenever sufficient PLB were available, responsiveness to CMVLA, CMVEA and all three mitogens was measured.

Stimulation of Patients' PBL with CMVLA, CMVEA and Mitogens

Individual responses of patients' PBL to CMVLA are shown in figure 1. The number of patients in each group with a positive SI was as follows: normal/mild 25 of 57 (44%); moderate dysplasia 15 of 35 (43%); severe dysplasia 26 of 46 (57%); and CIS 17 of 34 (50%). The difference in the percentage of positive patients in any group compared with any other was not statistically significant by the χ^2 test ($\chi^2 = 0.02$; $0.90 > p > 0.80$).

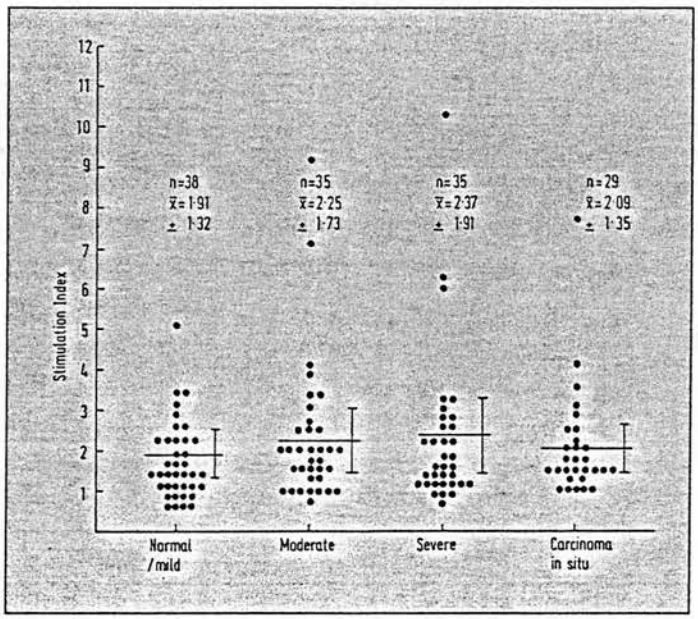

Fig. 2. Stimulation Index (mean ± SD) of PBL from groups of patients with grades of cervical dysplasia towards CMVEA.

Although the mean response to CMVLA seen in the normal/mild group of patients was lower than that of all other groups (fig. 1), the difference was not statistically significant by Student's t test (normal/mild versus moderate dysplasia, $t = 1.53$, $0.20 > p > 0.10$; versus severe dysplasia, $t = 1.24$, $0.30 > p > 0.20$; versus CIS, $t = 1.08$, $0.30 > p > 0.20$).

A total of 137 patients' PBL were tested for responsiveness towards CMVEA (fig. 2). There was no significant difference in either the percentage of positive responses in any group compared with another, or the mean SI values obtained.

The results of patients' PBL responses to mitogens are shown in table I where it can be seen that while the mean response to both PHA and PWM of patients within the CIS group was lower than that of others, the difference was not statistically significant by Student's t test ($p < 0.05$).

Correlation between SI and Antibody to CMV

Of the 172 patients whose lymphocyte responsiveness to CMV had been measured, ELISA was carried out on 159 corresponding sera which were available. The results of correlation between SI and the presence of CMV antibody are shown in table II. Correlation between positive SI induced by both CMVLA and CMVEA and the ELISA test for antibody was good. Of 81 tests positive by LS with CMVLA, 66 (82%) were also positive by ELISA. In addition, of 50 LS-positive tests obtained with CMVEA, 42 (82%) were also positive by ELISA. Table II also shows the correlation between ELISA and those tests negative by LS with both CMVLA and CMVEA. Of a total of 78 patients' PBL which did not respond in LS tests to CMVLA, 47 (60%) were seronegative by ELISA. Forty-two of 69 (61%) patients whose lymphocytes did not respond

Table I. Lymphocyte responses of patients to PHA, PWM and Con A

Patient group	SI to mitogens		
	PHA	PWM	Con A
Normal/mild dysplasia (n = 46)	24.10 ± 11.62 (0.2>p>0.1)	14.11 ± 8.87 (0.3>p>0.2)	19.40 ± 5.53
Moderate dysplasia (n = 41)	26.41 ± 10.53 (0.1>p>0.05)	14.88 ± 6.83 (0.2>p>0.1)	22.29 ± 8.51 (0.2>p>0.1)
Severe dysplasia (n = 43)	22.67 ± 9.22 (0.5>p>0.4)	15.11 ± 4.11 (0.1>p>0.05)	21.66 ± 8.49 (0.2>p>0.1)
CIS (n = 30)	20.88 ± 9.40	12.06 ± 4.05	19.55 ± 4.73

2×10^5 PBL were incubated for 6 days with PHA, PWM and Con A at final concentrations of 1:160, 1:40 and 0.3 μg/ml, respectively. The mean SI + SD responses of PBL from patients with CIS were compared to those from patients with other grades of dysplasia by Student's t test.

n = Number of patients.

Table II. Correlation between LS and ELISA to CMV antigens

Patient group	Number with positive ELISA / Number with positive LS to CMVLA	Number with positive ELISA / Number with positive LS to CMVEA	Number with negative ELISA / Number with negative LS to CMVLA	Number with negative ELISA / Number with negative LS to CMVEA
Normal/mild dysplasia	19/24 (79%)	11/13 (85%)	20/29 (69%)	17/21 (81%)
Moderate dysplasia	12/14 (86%)	12/14 (86%)	11/15 (67%)	8/15 (53%)
Severe dysplasia	22/26 (85%)	14/22 (79%)	9/19 (58%)	9/17 (53%)
CIS	13/17 (76%)	9/13 (78%)	9/15 (60%)	8/16 (50%)
Total	66/81 (82%)	41/50 (82%)	47/78 (60%)	42/69 (61%)

to CMVEA were also seronegative by ELISA.

Although there was good agreement between a positive LS result for CMI and the presence of CMV antibody, the correlation coefficient (r) between SI and antibody titre as measured by absorbance was not statistically significant ($r = 0.228$, $t = 1.36$, $0.1 > p > 0.5$).

Discussion

CMV has recently been tentatively proposed as a candidate virus implicated in the genesis of cervical cancer. Such an association was suggested because CMV has been isolated from cervical secretions [16] and cervical tumour biopsies [4]. In vitro transfection experiments have demonstrated the

ability of specific CMV DNA fragments, obtained after restriction endonuclease digestion, to induce transformation in NIH 3T3 target cells [17, 18].

Cervical cancer is thought to be a multistep disease beginning with a mild dysplasia and progressing, usually slowly, through moderate to severe dysplasia which may, unless treated, advance to CIS and thereafter to invasive carcinoma. If CMV is involved in any of these stages, specific viral antigens or virally induced antigens might be expressed on cervical cells. An immune response would thus indicate involvement of the virus.

In this work, a specific T-cell-mediated immune response was sought using the LS test. One CMV antigen preparation contained viral antigens synthesized late in a productive replication cycle. No group of patients exhibited an increased responsiveness to this preparation and the frequency of positive responders did not differ within groups.

Antigens synthesized 24–48 h after infection by CMV represent early, mostly non-structural virally induced proteins. These may include the protein(s) expressed during or necessary for cell transformation. In these assays, however, no group of patients responded to this second antigen preparation in a manner different from any other group.

CMI responses of all these patients as measured by lymphocyte responsiveness to specific CMV antigens correlated well with the patients' humoral antibody status demonstrated by ELISA. While there was 82% correlation between positive SI and the presence of CMV antibody, there was no correlation between the magnitude of the lymphocyte response and the levels of antibody in the patients' serum. A similar lack of correlation has previously been described in a comparison between LS and antibody detected by neutralization, indirect haemagglutination and CFTs [15].

A positive LS test depends primarily on the presence in the blood sample of a sufficient number of T cells with memory of CMV antigens. In vitro, these have to be presented with the antigen on monocytes, so that proliferation is initiated, followed by lymphokine production and recruitment of other lymphocytes. A number of factors can therefore influence the outcome of the LS test so that an immune individual might respond to the antigen preparation poorly and appear to be negative. In this study 40% of blood samples which were negative by LS were shown to possess CMV antibody by ELISA. On the other hand only 6% of those seronegative by ELISA gave a positive LS result. As a measure of prior exposure to CMV, therefore, the detection of antibody is more accurate than the demonstration of specifically sensitized lymphocytes. However, in the compromised host, circulating antibody does not necessarily confer immunity.

Although the present work did not demonstrate involvement of CMV in the various groups of patients studied, lymphocyte responses may be important in delaying the progress of the disease. The role of natural killer cells has already been examined [13] and presently under investigation is the response of interleukin-2-generated cytotoxic T cells towards dysplastic cells in culture.

Acknowledgements

We wish to thank Dr. *G. Smart* and Dr. *J. Livingston* of the Lothian Area Colposcopy Clinic for their help in this project, and Mrs. *M. Murray* for taking blood specimens from the patients. Financial support was provided by the Cancer Research Campaign.

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Received: March 11, 1985
Accepted: April 17, 1985

W.A. Neill,
Department of Bacteriology,
University of Edinburgh Medical School,
Teviot Place,
Edinburgh EH8 9AG (UK)

Copper and Zinc Levels in Serum from Patients with Abnormalities of the Uterine Cervix

Running headline: Serum Cu and Zn in Patients with CIN.

Ann Grail and Mary Norval

Department of Bacteriology

University of Edinburgh Medical School

Teviot Place

Edinburgh EH8 9AG

Correspondence to Dr A Grail, address as above.

INTRODUCTION

A variety of studies has reported an increase in the level of copper (Cu) in the serum of patients with malignancy. This increase has been found in carcinomas such as gastric (1), mammary (2), cervical (3), bladder (4) and bronchial (5); in sarcomas (6); and in haematopoietic malignancies (7,8). Several of these reports have suggested that the serum Cu level is a useful indicator of disease activity, response to treatment and recurrence of tumour.

About 90% of the Cu in serum is usually contained within the glycoprotein ceruloplasmin. Work by Linder (9) has indicated that increased synthesis of ceruloplasmin by the liver may be responsible for increased concentrations of ceruloplasmin and Cu in malignancy.

Although several of the above studies have also described changes in serum zinc (Zn)levels in human cancer patients, the relationship appears less straightforward than that for Cu. Generally, subnormal Zn levels are found in patients with malignancy and there is a return to normal values with favourable response to therapy (7). Since it appears that the serum Cu level is influenced by the level of Zn because these metals are physiological antagonists (10), the Cu:Zn ratio has been advocated as a more useful measurement of disease activity. However, some workers have reported little or no alteration of serum Zn in malignancy (11, 12) or increased levels (6, 13).

Recently, there has been further indication of the potential

value of serum Cu and Zn estimations in gynaecological tumours (12). Six cases of cervical carcinoma were included in this study and it was suggested that low-stage malignancy could not be diagnosed by this method. The present investigation was undertaken to establish whether changes in Cu and Zn levels in the serum occurred with progressive stages of cervical dysplasia, since dysplasia is presumed to precede carcinoma-in-situ and invasive carcinoma.

MATERIALS AND METHODS

Patients and samples

Blood samples were obtained from women attending Gynaecology Out-Patient Departments at the Elsie Inglis Hospital and Royal Infirmary, Edinburgh. Serum was separated and stored at $-20^{\circ}C$. Red cells have a high Zn content (14), hence any haemolysed specimens were rejected. In addition, patients known to have liver disease (15) or those receiving oral contraceptives (16) were excluded as this may alter serum Cu and Zn levels. Routine pathological examination was carried out on several sections of cervical biopsies taken at the time of the blood sample. Twenty-one patients were classified as having no evidence of cervical dysplasia, 24 patients had mild dysplasia CIN I (cervical intraepithelial neoplasia - grade 1), 21 patients had moderate dysplasia, CIN II, 65 patients had severe dysplasia/carcinoma-in-situ, CIN III, and 9 patients had invasive carcinoma (Table 1).

Measurement of serum Cu, Zn and ceruloplasmin

Serum Cu and Zn concentrations were determined on the SP9 atomic absorption spectrophotometer with SP9 computer (Pye Unicam) using air-acetylene flame (17). The concentration of Cu and Zn was determined in pooled serum by standard additions analysis (18). To 10 ml of pooled serum 100 ul of Cu standard solutions (5, 10, 50, 100, 250, 500 mg/ml) and 100 ul of Zn standard solutions (5, 10, 50, 100, 300 mg/ml) were added and thoroughly mixed. Using the values for these sets of standards and blanks, the Cu and Zn concentrations of the

pooled serum and unknown serum samples could be calculated. All standards and sera were measured in triplicate and the mean value estimated. An absorbance versus concentration line was plotted; the intercept of this straight line on the concentration axis gave the concentration of the metal in the pooled serum used to make up the standards, and the concentration of the metal in the test sera could then be calculated. The stability of the system was routinely checked after every 15 measurements. Standards were cupric nitrate and zinc chloride standard solutions "spectrosoL" (BDH Chemicals Ltd.). New standards were always prepared on the day of each analysis.

Serum ceruloplasmin was estimated by determining the serum copper oxidase activity by the method of Ravin as described by Varley (19).

RESULTS

Patients and Samples

Serum samples and cervical biopsies were obtained from 140 patients. They were not taking oral contraceptives, were not pregnant and were apparently healthy in other respects. Routine pathology on several paraffin sections of the biopsies classified the patients into groups depending on the extent of dysplasia and the presence of in-situ carcinoma and invasive carcinoma. Where difficulty in the grading of some specimens made the distinction between two dysplastic groups less clear-cut, these specimens were put in the higher dysplastic group. Similarly, distinction between severe dysplasia and carcinoma-in-situ is difficult; hence these samples were classifed together as CIN III. The age range of patients in each group was similar, as is the mean age (shown in Table 1).

Estimation of serum Cu, Zn and ceruloplasmin

Procedures involving precipitation of serum samples, e.g. by trichloroacetic acid and extraction of samples have been shown to be influenced by volume errors, errors resulting from incomplete extraction and problems of contamination (17). Dilution methods may reduce sensitivity and precision and the risk of contamination is increased (20). Therefore, because the physicochemical properties of the standards are as similar as possible to the samples (21), the method of standard additions analysis was chosen for estimation of serum Cu and Zn in undiluted serum (18).

The average serum Cu level in healthy females was found to be

125 ug/dl (SD ± 0.16). In subjects with dysplasia the mean serum Cu level increased: CIN I 133 ug/dl (SD ± 0.19) $p < 0.5$, CIN II 141 ug/dl (SD ± 0.24) $p < 0.02$, CIN III 140 ug/dl (SD ± 0.28) $p < 0.05$. In the 8 subjects with invasive carcinoma the mean serum Cu also increased to 147 ug/dl (SD ± 0.26) $p < 0.02$. These results are shown in Fig. 1.

In general, the average serum Zn levels were decreased in dysplasia and in invasive carcinoma when compared to the mean serum Zn value for healthy individuals 102 ug/dl (SD ± 0.34). The mean serum Zn values were: 81 ug/dl (SD ± 0.19) $p < 0.02$ for CIN I; for CIN II 90 ug/dl (SD ± 0.28) $p < 0.5$; for CIN III 93 ug/dl (SD ± 0.28) $p < 0.5$; for invasive carcinoma 73 ug/dl (SD ± 0.12) $p < 0.05$ (Fig. 2).

Calculation of the Cu:Zn ratio provided greater differences in the mean values for normal and dysplastic states and invasive carcinoma. However, increased variation within each group was apparent. Mean Cu:Zn values were:1.36 (SD ± 0.57) for healthy individuals, 1.72 (SD ± 0.44) $p < 0.05$ for patients with CIN I, 1.72 (SD ± 0.41) $p < 0.05$ for patients with CIN II, 1.61 (SD ± 0.46) $p < 0.1$ for patients with CIN III and 2.07 (SD ± 0.51 $p < 0.01$ for invasive carcinoma (Fig. 3).

Measurement of serum ceruloplasmin showed little differences between normal and dysplastic groups CIN II, CIN III and invasive carcinoma. Mean ceruloplasmin values were: 0.48 (SD ± 0.09) for healthy females, 0.49 (SD ± 0.12) for patients with CIN II, 0.5 (SD ± 0.18) for patients with CIN III and 0.56 (SD ± 0.16) for patients with invasive carcinoma. There was a marked decrease in serum ceruloplasmin in patients with CIN I, 0.4 (SD ± 0.09) $p < 0.01$, (Fig. 4).

DISCUSSION

The literature regarding the elevation of serum Cu in malignancy generally indicates an increase in Cu levels with increasing disease activity, a decrease in response to treatment and increasing levels in cases of relapse (1-8). It has been suggested that increased serum Cu and ceruloplasmin levels in malignancy may play a role in host defense mechanisms (22). In addition or alternatively, Cu may be important for increased production of essential respiratory enzymes (23).

Increased serum Cu levels were reported in women with cervical carcinoma by O'Leary and Feldman (3) and the degree of elevation of serum Cu was related to the stage of carcinoma. In the present study there appeared to be a progressive increase in the mean serum Cu value with increasing dysplasia of the cervix and invasive carcinoma (Fig. 1). However, large overlapping of values from all the groups makes it unlikely that this finding will be important in early diagnosis before there is pathological evidence of carcinoma.

Although ceruloplasmin levels generally reflected serum Cu values, there was no significant difference between CIN II, CIN III and invasive carcinoma and the group with no evidence of dysplasia (Fig. 4). An unexpected finding was a decrease in ceruloplasmin in patients with mild dysplasia, CIN I. It is possible that the measurement of ceruloplasmin may provide a less sensitive means of estimating Cu activity. The linear relationship between ceruloplasmin and total serum Cu may not always apply in particular disease states, and it remains to be

confirmed that oxidase activity and Cu concentrations are equivalent (24).

Decreasing mean serum Zn concentrations were observed in dysplasia and invasive carcinoma of the cervix when compared to the control group (Fig. 2). These differences were significant for CIN I and invasive carcinoma groups. In general, subnormal serum Zn levels have been reported in cancer patients and a return to normal levels has been associated with a favourable prognosis (7). It is thought that rapidly dividing tumour tissue has an increased demand for Zn, which is important for nucleic acid metabolism and protein synthesis (25). However, as there was considerable overlap between the Zn values in the present study, Zn estimation on its own would not allow definition of the malignant state.

Several studies have advocated the use of the Cu:Zn ratio in prognosis and management of patients with malignancy (6,7). In the present study the serum Cu:Zn ratio gave a more significant difference between the stages of dysplasia and invasive carcinoma and the control group than individual serum Cu or serum Zn levels (Fig. 3). Brandes et al (12) found that the serum Cu:Zn ratio discriminated the cancer patients from normal individuals better than measurement of Cu or Zn alone in their study of 25 patients with malignant gynaeological tumours. They consider that the increased serum Cu:Zn ratio was mainly due to increased serum Cu and, that in patients with advanced stages of tumour, a contributory effect of a decrease in serum Zn occurred. Similarly, in the present study

increased Cu:Zn ratios largely reflected an increase in the serum Cu levels and the additional effect of lower Zn levels was apparent mostly in invasive carcinoma. On the other hand, Capel et al (26) reported no increased Cu nor decreased Zn in sera from 11 patients with tumours of the cervix, and the mean Cu:Zn ratio was similar to that for healthy controls. Fisher et al (22) found that, although serum Cu levels were generally elevated in patients with melanoma and this elevation was related to the degree and extent of tumour activity, serum Zn levels and hence the Cu:Zn ratio did not reflect tumour activity.

Although it would be tempting to speculate that pre-malignant changes in serum Cu levels were observed in the present study, the minor differences in the pathological staging of cervical dysplasia and the large overlapping of values in all the groups make this unlikely. Increased serum Cu and decreased serum Zn are not exclusive to malignant disease and can occur in acute and chronic infections, chronic liver diseases and pregnancy (27). Although most of these conditions were excluded in the present study, occult infection cannot be discounted. Tissue levels of trace metals appear to vary not only with age, sex and other factors but particularly with the organ of origin (28). A similar study on the levels of Cu and Zn in normal, dysplastic and malignant cervical tissue would perhaps reveal whether changes of Cu in the serum directly reflect tissue alterations of the metals and precede pathological evidence of malignant cells.

We acknowledge the very kind help of Dr G. Smart of the Royal Infirmary, Edinburgh, Mrs M Murray of the Department of Bacteriology and Dr A. Rowley of the Department of Chemistry, University of Edinburgh. This work was funded by the Cancer Research Campaign.

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	No. of Patients	Age Range (years)	Mean Age
No evidence of dysplasia	21	25 - 63	39
CIN I	24	24 - 51	33
CIN II	21	22 - 49	32
CIN III	65	22 - 49	35
Invasive carcinoma	9	27 - 60	38

Table 1: Classification of patients according to pathological resection of cervical biopsy specimens.

LEGENDS TO THE FIGURES

Fig 1. Serum copper levels (µg/dl) in women with various grades of cervical dysplasia up to invasive carcinoma.

Fig 2. Serum zinc levels (µg/dl) in women with cervical dysplasia and with invasive carcinoma.

Fig 3. Serum Cu/Zn ratios in women with cervical dysplasia and with invasive carcinoma.

Fig 4. Serum ceruloplasmin levels (O.D.) in women with cervical dysplasia and with invasive carcinoma.

Archives of Virology
O/49

Archives of Virology ■, ■■–■■ (1986)

Effect of Concanavalin A and Succinyl Concanavalin A on Cytomegalovirus Replication in Fibroblasts

By

Ann Grail and Mary Norval

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, U.K.

With 6 Figures

Accepted February 10, 1986

Summary

In order to investigate inhibition of viral replication, human embryonic fibroblasts infected with cytomegalovirus (CMV) were treated with 0 to 25 μg/ml concanavalin A (Con A) and 0 to 150 μg/ml succinylated Con A (S-Con A). Alteration in cellular morphology occurred by day 2 post infection (p. i.) in cultures treated with 10 μg/ml Con A and 25 μg/ml S-Con A. With increasing concentrations of Con A and S-Con A there was decreased virus production from day 4 p. i. to day 10 p. i. Increasing levels of Con A and S-Con A also reduced the number of cells in culture. When virus titres were corrected to take cell number into account, decreased CMV titres in Con A and S-Con A treated cells appeared mainly to reflect decreased cell numbers. In support of this finding, in comparison with untreated CMV-infected fibroblasts, viral DNA synthesis was reduced and acid phosphatase levels were increased in CMV-infected cells treated with Con A or S-Con A.

Introduction

The plant lectin, concanavalin A (Con A) specifically binds to cell surfaces containing α-D-mannopyranosyl and α-D-glucopyranosyl residues (12, 14). However, following infection by a wide range of viruses (4, 13) or transformation (18), the receptors are redistributed into clusters. As a result both virus infected and transformed cells are more easily agglutinated with Con A (9).

Succinylation of Con A converts the molecule from a tetrameric to a dimeric form which apparently reduces the crosslinking capacity (7). Con A and S-Con A, therefore, have different biological activities and are both useful for investigating how cell surface related changes may influence intracellular behaviour.

When cells are productively infected with herpes simplex virus (HSV) (17), or cytomegalovirus (CMV) (10, 16), they develop an affinity for the Fc portion of immunoglobulin. These Fc receptors appear to be viral gene products (3). In the case of HSV-infected cells, Fc receptors share some measure of identity with receptors for anti-HSV-antibody and Con A; inhibition of viral replication occurred in the presence of Con A (1). In addition, inhibition of HSV-replication in cultures treated with S-Con A has been reported (5).

In this paper we report on the results of similar studies using CMV-infected cells treated with Con A and S-Con A, with a view to investigating how cell surface interactions may alter viral replication and perhaps play a role in the latency of CMV.

Materials and Methods

Virus Growth Studies

Strain AD 169 of CMV was used throughout (8). Human embryonic fibroblasts (HEF) at pass 3 to 10 were grown in modified Eagle's medium supplemented with 5 per cent newborn calf serum (NBCS), 100 i. u./ml penicillin and 200 µg/ml streptomycin. When confluent, cells in 24-well culture plates (Flow Laboratories) were infected with CMV at a multiplicity of infection of 1 to 2, at 37° C for 1 hour. The cell monolayers were washed with phosphate-buffered saline (PBS) and maintained in medium containing 2 per cent NBCS. Con A (Sigma) at 0, 5, 10, 15, 25 µg/ml or S-Con A (Sigma) at 0, 10, 25, 50, 100, 150 µg/ml were added to cultures at this time.

Cells were harvested at daily intervals by scraping into the medium with a rubber policeman. After freeze-thawing, and ultrasonification at 9 Kc/s for 2 minutes, the virus was titrated in HEF cells with a 0.25 per cent agarose (Seaplaque, FMC, U.S.A.) overlay and the titre was expressed as $\log_{10}$ plaque forming units (PFU)/ml. Similarly infected cultures containing the same concentration of lectin were washed with PBS, the cells removed with PBS containing 0.1 per cent trypsin, 0.01 per cent versene and the cell number was determined with a haemocytometer. Corrected virus titres were expressed as $\log_{10}$ (PFU/10^6 cells).

DNA Synthesis

Confluent uninfected and AD 169-infected HEF monolayers (about 2×10^6 cells) were incubated with 0, 10, 25 µg/ml Con A or 0, 10, 25, 50 µg/ml S-Con A until the cytopathic effect was well advanced. The medium was replaced with fresh medium containing the same concentration of lectin and 2 µCi/ml(methyl-^{3}H) thymidine (specific activity, 84 Ci/mmol; Amersham) and the cells incubated for a further 3 days at 37° C. Infected and uninfected cells were harvested using glass beads. The cells were washed three times in 0.01 M Tris HCl buffered saline pH 7.4, resuspended in 9 ml buffer and digested with 1 per cent SDS in lysis buffer (0.05 M Tris, 0.01 M EDTA pH 8.0) containing 0.2 mg/ml proteinase K (Sigma) for 4 hours at 37° C. DNA was extracted twice with redistilled phenol, saturated with TES buffer (0.05 M Tris, 0.005 M EDTA, 0.15 M NaCl pH 7.4), and precipitated with ethanol. The sample was then redissolved in TES buffer, suspended in TES buffered CsCl with a density of 1.72 g/ml and centrifuged for 68 hours at 100,000 g with a Beckman SW 50.1 rotor at 20° C. Fractions were collected, the refractive index measured with a refractometer, and then precipitated with trichloracetic acid (TCA). The precipitate was collected on glass fibre discs, washed with 10 per cent TCA followed by methanol, and after drying the discs were counted in toluene-based scintillation fluid.

Acid Phosphatase Activity

HEF were infected with AD 169 as described previously and incubated at 37° C in the presence of Con A at 0, 10, 25 µg/ml or S-Con A at 0, 10, 25, 50 µg/ml. Each concentration was tested in four cultures for each time interval. At the indicated times post infection, two of the four cultures were trypsinized and the cells counted; extracts were prepared from the other two cultures by the procedure outlined in (6). Briefly, the cell monolayers were washed three times with PBS and suspended in 0.1 ml distilled H_2O. After overnight storage at −30° C, the cultures were thawed and 0.3 ml 0.4 M sodium acetate buffer pH 5.0 and 0.3 ml 0.2 M B-glycerophosphate (pH 5.0) were added. Incubation was carried out at 37° C for 2 hours and the reaction stopped by the addition of 0.4 ml 20 per cent TCA. The solution was clarified by centrifugation at 2000× *g* for 10 minutes and analyzed for inorganic phosphate (Pi) (2). Acid phosphatase activity was corrected for cell number and is expressed as mg Pi/10^6 cells.

Effect of S-Con A and Con A on CMV Replication in HEF

HEF cultures were infected as described in Materials and Methods and maintained in the presence of S-Con A (at 0, 10, 25, 50, 100 150 µg/ml) or Con A (at 0, 5, 10, 15, 25 µg/ml) from 1 hour post infection until the time of harvest. The virus produced was then titrated in HEF.

The addition of S-Con A and Con A altered the morphology of the infected cell monolayers. By two days post infection (p. i.) untreated fibroblasts appeared virtually unaltered by the presence of virus. However, at the same time p. i. a proportion of cells had rounded in cultures treated with 10 µg/ml Con A and there were large gaps in the cell monolayer. This effect was aggravated in the presence of 25 µg/ml Con A. Similar effects were observed with S-Con A. Therefore, cell numbers were determined in cultures at each concentration of lectin by trypsinizing and counting cells in parallel cultures daily for 10 days p. i. Measurement of cell numbers in such a collection of cultures gives, at best, an estimation which will vary from one experiment to another. Increasing concentrations of S-Con A and Con A reduced the number of cells in culture; concentrations of Con A greater than 25 µg/ml and S-Con A greater than 50 µg/ml reduced cell numbers to such an extent that estimation of cell number over the period of infection was not reliable (data not shown).

In cultures infected with CMV, production of virus occurred after an eclipse period of approximately 3 days and a sharp increase in virus titre was seen by day 4 p. i. (Figs. 1 and 2). There was another increase after day 8 presumably due to a second round of replication in the cells not infected initially. With increasing concentrations of Con A, the eclipse period was unchanged but the amount of virus produced on day 4 and on subsequent days was reduced (Fig. 1 a).

In the S-Con A treated cultures a similar reduction in virus titre was seen at day 4—5 p. i. compared with the control culture (Fig. 2 a). The second burst of CMV activity was more obvious in these cultures and occurred from day 7—9 p. i. even in the presence of S-Con A, although to a lesser extent. By day 10 inactivation of virus was apparent.

However, when virus titre was corrected by taking differences in cell number attributable to Con A and S-Con A treatment into account, and expressed as PFU/10^6 cells, very little effect of Con A or S-Con A on CMV replication was apparent (Figs. 1 b and 2 b).

DNA Synthesis in CMV-infected HEF Treated with Con A and S-Con A

There was very little difference in the incorporation of ^{3}H-thymidine in uninfected cultures treated with concentrations of S-Con A ranging from 10 to 50 µg/ml or Con A from 10 to 25 µg/ml compared with untreated fibroblasts (data not shown). In CMV-infected cultures, although treatment with S-Con A at 10 and 25 µg/ml only slightly altered the amount of viral and cellular DNA, in the presence of 50 µg/ml S-Con A a much greater decrease in incorporation of ^{3}H-thymidine occurred (Fig. 3). There appeared to be a single peak of DNA but the width and the range in density of the peak suggests that both viral and cellular DNA was present. On the other hand, CMV-infected cultures treated with 10 and 25 µg/ml Con A gave only one relatively sharp peak of DNA and this was probably cellular DNA, having a density of 1.70 g/ml approximately (Fig. 4).

Acid Phosphatase Activity in CMV-infected Cells Treated with Con A and S-Con A

CMV-infected HEF were incubated with various concentrations of Con A or S-Con A over 9 days. Extracts prepared from cultures were analyzed for inorganic phosphate (an indirect measurement of acid phosphatase activity)

and at the same time, duplicate cultures were used to estimate cell number. Before correction for cell number, no significant differences in acid phosphatase levels were apparent in CMV-infected cultures treated with Con A and S-Con A (data not shown). However, when acid phosphatase activity is expressed as mg Pi/10^6 cells, increasing levels were associated with increasing concentrations of Con A and S-Con A (Figs. 5 and 6). Increases in acid phosphatase activity occurred 3 and 7 days after infection in S-Con A treated cells (Fig. 5), which coincided with increased virus titres on these days (Fig. 2). Decreasing levels on day 5–6 and day 9 probably reflect lysis of the cells. Similar increases in acid phosphatase activity occurred in Con A treated cells and much larger levels were apparent later in infection (Fig. 6).

Discussion

It has been suggested that binding of Con A (1) and S-Con A (5) to HSV-infected cells inhibits production of infectious virus. This effect may provide one method of studying mechanisms of latency of Herpes viruses by determining how viral replication is halted. We treated CMV-infected fibroblasts with Con A and S-Con A but found that decreased CMV titres reflected decreased cell numbers. Alterations in cellular morphology were noticeable even at the lower levels of lectin employed. In support of this finding similar changes have also been reported in RK-13 cells infected with vesicular stomatitis virus and treated with Con A (15).

GARRITY *et al.* (5) suggested that inhibition of viral replication in HSV-infected cells treated with S-Con A did not occur at the level of viral DNA synthesis. In contrast, we found a reduction in viral DNA in CMV-infected cultures treated with S-Con A, and this reflected a decrease in the number of viable cells capable of supporting CMV infection. The effect of Con A at 10 µg/ml was more remarkable in that viral DNA did not seem to be present (Figs. 4 b and c). The amount of virus is reduced 30-fold (Fig. 1 a) in the presence of 10 and 25 µg/ml Con A, compared with untreated infected cells. If the amount of viral DNA synthesis in untreated cells is reduced by this factor then counts per minute in the region of 10^2 or lower would be expected in the presence of Con A, and this may not be sufficient for detection. In addition, although viral DNA synthesis was detected in the presence of 50 µg/ml S-Con A, this probably reflects 10 times more viral replication occurring in S-Con A treated cells (Fig. 2 a) than in Con A treated cells (Fig. 1 a).

GARRITY *et al.* (5) considered that decreased HSV replication in S-Con A treated cells could be caused by destruction of virus by lysosomal enzymes, although this could not be demonstrated in practice. However, in the CMV system, we found that, in addition to correcting for the virus growth curve, correction of acid phosphatase levels taking cell numbers into account, revealed an increase in acid phosphatase activity with increasing concentrations of Con A and S-Con A. This increased activity could destroy virus particles but presumably cell lysis occurs before infectious virus can be produced.

Decreased synthesis of viral polypeptides may be involved in the inhibition of HSV-replication in cells treated with S-Con A (5). On the other hand, KHELIFA and MENZES (11) concluded that Con A acted on the early stages of Epstein-Barr virus replication at the cell or virus surface. Their findings suggest that inactivation of extracellular virus by Con A would decrease the virus titre. Although CMV is very cell-associated, strain AD-169 is highly tissue culture adapted, and ~~at~~ virus spreads via the medium as well as by cell-to-cell contact. Thus extracellular inactivation of CMV by lectins may have occurred in our system. However, such low levels of lectin would not saturate cellular binding sites, and it is unlikely that inhibition of CMV replication would mainly result from inactivation of extracellular virus or the blocking of virus from entering cells.

Taking together the morphological evidence, differences in cell numbers, correction of CMV titres when cell numbers are considered, reduced viral DNA synthesis, and increased acid phosphatase activities, decreased CMV titres in infected cultures treated with Con A and S-Con A predominantly reflect a reduction in cell viability and not a specific effect on any one stage of the viral replication cycle.

Acknowledgements

This work was supported by the Cancer Research Campaign.

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Authors' address: Dr. Ann Grail, Department of Veterinary Medicine, Langford House, Langford, Bristol BS 18 7 DU, U.K.

Received October 31, 1985

Printed in Austria

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Fig. 1. Virus production in CMV-infected cultures treated with Con A. *a* Virus titre expressed as $\log_{10}$ PFU/ml. (●) 0 Con A; (□) 5 µg/ml Con A; (▲) 10 µg/ml Con A; (■) 15 µg/ml Con A; (○) µg/ml Con A. *b* Virus titre corrected for cell number, symbols as for *a*

Fig. 2. Virus production in CMV-infected cultures treated with S-Con A. *a* Virus titre expressed as $\log_{10}$ PFU/ml. (●) 0 S-Con A; (▲) 10 µg/ml S-Con A; (■) 25 µg/ml S-Con A; (○) 50 µg/ml S-Con A; (△) 100 µg/ml S-Con A; (□) 150 µg/ml S-Con A. *b* Virus titre corrected for cell number, symbols as for *a*

Fig. 3. CsCl buoyant density analysis of DNA from CMV-infected cells treated with S-Con A. (All fractions were counted but in the graphs every second fraction is represented from areas outwith the peak fractions). *a* DNA from CMV-infected cells. DNA from CMV-infected cells treated from 1 hour p. i. with: 10 µg/ml S-Con A *(b)*; 25 µg/ml S-Con A *(c)*; 50 µg/ml S-Con A *(d)*

Fig. 4. CsCl buoyant density analysis of DNA from CMV-infected cells treated with Con A. (All fractions were counted but in the graphs every fraction is represented from areas outside the peak fractions). *a* DNA from CMV-infected cells. DNA from CMV-infected cells treated from 1 hour p. i. with: 10 µg/ml Con A *(b)*; 25 µg/ml Con A *(c)*

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Fig. 5. Acid phosphatase levels (mg P. i./10^6 cells) in CMV-infected cells treated with S-Con A. (●) 0 S-Con A; (□) 10 µg/ml S-Con A; (■) 25 µg/ml S-Con A; (○) 50 µg/ml S-Con A

Fig. 6. Acid phosphatase levels (mg P. i./10^6 cells) in CMV-infected cells treated with Con A. (●) 0 Con A; (□) 10 µg/ml Con A; (■) 25 µg/ml Con A

Nature Vol. 264 November 11 1976

Experimental production of sheep pulmonary adenomatosis (Jaagsiekte)

SHEEP pulmonary adenomatosis (SPA) or Jaagsiekte is a contagious disease of unknown aetiology[1]. A herpesvirus has been isolated from such tumours in Britain[2] and in Africa[3,4] but attempts to produce adenomata with this virus have been unsuccessful (ref. 5 and W.B.M., G. Robinson, and K.W.A., in preparation). More recently, evidence has been presented that a type C virus with a density on a sucrose gradient of 1.5–1.20 g ml^{-1}, containing 60–70S RNA and RNA-directed DNA polymerase (reverse transcriptase) is present in the adenomatous tissue[6]. We wish to describe the production of SPA tumours in sheep experimentally infected with both agents.

Techniques[7] similar to those of Perk *et al.*[8], were used to confirm the presence of a reverse transcriptase-producing (RTP) agent in SPA tumour tissue from Scottish sheep. This enzyme was not detected in similar fractions from the lungs of unaffected sheep (Table 1).

A suspension of tumour from a natural case of SPA was subjected to equilibrium density centrifugation on a sucrose gradient. Fractions with densities of 1.15–1.17 g ml^{-1} were centrifuged at 150,000*g* for 60 min at 4 °C and the pellet,

Table 1 DNA polymerase activity of fractions of tumour or unaffected lung

Sheep no.	Clinical state	Reagent added to standard reaction mixture: No addition	+Poly(A)·oligo(dT)$_{10}$	+Actinomycin D
A525	Adenomatosis	60* (1)‡	930 (15)	70 (1.2)
J2	Adenomatosis	150 (1)	370 (2.5)	33 (0.2)
J4	Adenomatosis	40 (1)	330 (8.2)	26 (0.7)
J13	Adenomatosis	110 (1)	800 (7.3)	115 (1.1)
J14	Adenomatosis	153 (1)	750 (4.9)	133 (0.9)
N1	Unaffected	120 (1)	155 (1.3)	10 (0.09)
N15	Unaffected	117 (1)	173 (1.5)	47 (0.4)
N16	Unaffected	170 (1)	313 (1.8)	93 (0.6)

*Polymerase activity of 1.15–1.17 g ml^{-1} fraction from sucrose density gradients of tumour or lung tissue suspensions, measured as increase in c.p.m. ^{3}H-TTP incorporated into acid-insoluble material in 20 min using the methods outlined by Norval *et al*[7].

‡Ratio of comparative activities are given in parentheses and the discriminatory effect of poly(A)·oligo(dT)$_{10}$ and actinomycin D on polymerase activity in the adenomata and healthy tissues are shown.

resuspended in 199 medium, was injected endobronchially into six Cheviot sheep. Herpesvirus isolated from an SPA tumour and passaged four times in embryo sheep kidney cells and once in sheep pulmonary macrophages (titre $\geqslant 10^{6.7}$ TCD_{50} ml^{-1}) was similarly injected into four sheep. Both the herpesvirus and the density gradient fraction, containing the RTP agent, were injected into six more sheep. As controls, four sheep were given a supernate of tumour suspension and four, from the same flock, a supernatant prepared in an identical manner from apparently normal lung. To avoid cross infection, the groups of sheep were housed, as in previous transmission experiments, in separate loose boxes.

Results are shown in Table 2. One sheep, given the tumour supernatant, was clinically affected with SPA 6 months after inoculation. This diagnosis was confirmed histologically and all sheep were killed therefore 7–8 months after inoculation. A minimum of ten standard sites from the lungs of each sheep were examined histologically. In the group given the RTP agent alone, one sheep had two small (3–5-mm) adenomata and one other, histological evidence of adenomatous changes. Lesions in both sheep were minimal and present in four (13%) of a total of thirty-one sites examined. Of the sheep given both the RTP agent and the herpesvirus, three had definite macroscopic tumours and one was histologically positive (Fig. 1). No adenomatous lesions were found in any other sheep. All lungs were assayed for particles with reverse transcriptase activity and these were detected in lung tissue from two sheep (Table 2). Herpesvirus, which we have isolated from 24% of SPA tumours over the past 5 yr, was not recovered from any lung.

Table 2 Evidence of pulmonary adenomata in the inoculated sheep

Material inoculated	No. in group	Macroscopic tumours	Histological adenomatous changes	Number with reverse transcriptase activity
Tumour supernatant	4	1	2	1
Herpesvirus and reverse transcriptase-producing agent	6	3	4	1
Reverse transcriptase-producing agent	6	1	2	0
Herpesvirus	6	0	0	0
Normal lung supernatant	4	0	0	0

Suspensions of finely minced tumour or lung tissue were prepared in Eagle's medium containing antibiotics and centrifuged at 12,000*g* for 30 min at 4 °C (inocula of "tumour or normal lung supernatant"). The supernate was recentrifuged at 120,000*g* for 90 min and the resulting pellet gently resuspended in 1 ml TNE buffer, layered on to a preformed gradient of 20–70% sucrose prepared in TNE buffer and centrifuged in a Beckman SW 40 rotor at 85,000*g* for 16 h at 4 °C. Fractions between densities of 1.15–1.17 g ml^{-1} (the density of RNA tumour viruses) were pooled, diluted in TNE buffer and centrifuged at 150,000*g* for 60 min at 4 °C. The pellet obtained was suspended in 0.15 ml of TNE buffer and assayed for reverse transcriptase or further diluted for inoculation of sheep. Each inoculum was injected endobronchially in 8-ml volumes.

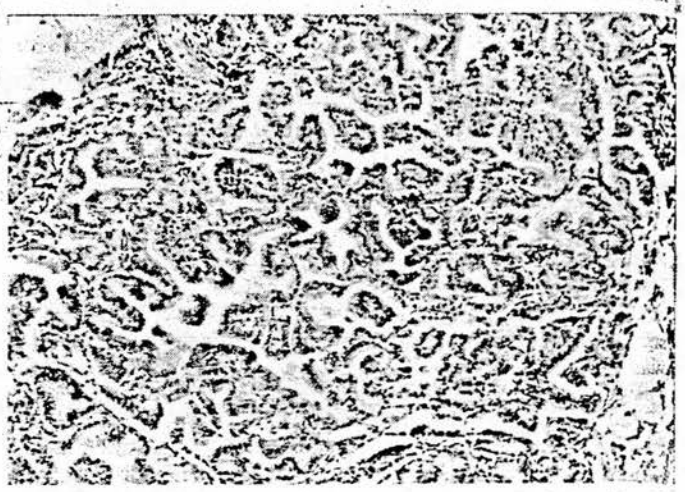

Fig. 1 Focus of adenomatous change.

We can find no evidence to support the view that these tumours arose adventitiously or were caused by myoplasma or other cultivable microorganisms. Since some sheep in both of the groups given the density fraction containing the RTP agent developed adenomatous lung lesions within 8 months of inoculation, it seems certain that this agent was involved in the production of these changes. Whether it alone is capable of initiating adenomatous changes or requires the assistance of the herpesvirus is conjectural and remains to be established. In the four affected sheep given both herpesvirus and RTP agent, lesions were extensive, and present in a mean of 63% of the areas of lung examined. This may indicate that the herpesvirus was involved in the production of these changes. The possibility that a small number of herpesvirus particles was present in the fraction containing the RTP agent cannot be dismissed completely and such contamination would simulate, to a lesser extent, the combined inoculum of herpesvirus and RTP agent. It may be that both viruses act synergistically to cause oncogenic transformation of the target cells, the type II alveolar cells or the cells of Clara in the bronchioles[4], but the precise role of these viruses can only be elucidated by further experiments with purified virus.

WILLIAM B. MARTIN
FRANK M. M. SCOTT
JAMES M. SHARP
KENNETH W. ANGUS
Animal Diseases Research Association, Moredun Institute, Edinburgh EH17 7JH, UK

MARY NORVAL
Virus Research Laboratory, Department of Bacteriology, Edinburgh University Medical School, Edinburgh, UK

Received August 10; accepted September 14, 1976.

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Br. J. Cancer (1978) **37**, 269

ANTITUMOUR RESPONSES INDUCED BY SHORT-TERM PRETREATMENT WITH TUMOUR CELLS

K. JAMES, R. T. CULLEN, I. MILNE AND M. NORVAL*

*From the Departments of Surgery and *Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland*

Received 19 September 1977 Accepted 11 October 1977

Summary.—The injection (s.c. or i.p.) of 10^6 live or lethally irradiated methylcholanthrene-induced fibrosarcoma cells into CBA/Ca mice one or 2 days before i.v. challenge with the same tumour inhibited the formation of artificial lung tumour metastases. In addition, it also frequently enhanced the cytostatic effect of peritoneal exudate cells on monolayers of the same tumour. The effects on lung tumour metastasis were not noted if X-irradiated tumour was injected i.v., or if s.c. administration was delayed until one day after i.v. challenge. Similar effects on tumour growth were also observed in C3Hf/Bu mice and (CBA/Ca × A/HeJ) F_1 hybrids which were pretreated (s.c.) with tumour shortly before i.v. challenge with the same tumour. Further studies in CBA/Ca mice suggested that the protective effect was tumour-specific, for the growth of i.v. injected tumour was not significantly inhibited by pretreatment with a number of other MC-induced or spontaneous tumours from the same and different strains.

A CONSIDERABLE number of investigations undertaken during recent years have shown that factors which suppress the function of lymphoreticular endothelial cell may be released by or extracted from a variety of tumours. For example, factors have been reported which suppress (a) the *in vitro* response of spleen cells to mitogens and alloantigens (De Lustro and Argyris, 1976) and sheep erythrocytes (Wong, Mankovitz and Kennedy, 1974; Kamo *et al.*, 1975); (b) macrophage chemotaxis *in vitro* (Meltzer and Stevenson, 1977*a*, *b*; Normann and Sorkin, 1977; Snydermann and Pike, 1976; Pike and Snydermann, 1976) and *in vivo* (Snyderman and Pike, 1976; Pike and Snyderman, 1976); (c) macrophage-mediated resistance to infection (North, Kirstein and Tuttle, 1976*a*, *b*); and (d) the *in vivo* response to syngeneic (North *et al.*, 1976*b*; Pike and Snyderman, 1976; Nelson and Nelson, 1977) and allogeneic (Bonmassar *et al.*, 1973) tumour transplants. It would also appear that the inhibitory effects observed *in vivo* are not always dependent upon the presence of a large tumour mass, for they may be evident within 24 h of the s.c. transplantation of 10^6 tumour cells (North *et al.*, 1976*a*, *b*).

The observed effects on macrophage function have led to the suggestion that neoplastic cells may abrogate or interfere with the early phases of immunosurveillance by releasing products which modify the localization and/or activity of macrophages (Snyderman and Pike, 1976; Pike and Snyderman, 1976; North *et al.*, 1976*a*, *b*; Nelson and Nelson, 1977). In view of the above suggestions and the observations of North *et al.* (1976*a*, *b*), we thought it was important to establish whether the injection of syngeneic tumour cells shortly before i.v. challenge with the same tumour cells would increase the incidence of artificial metastases, or interfere with the capacity of macrophages to exert a cytostatic effect on syngeneic target cells *in vitro*. Contrary to expectations we found that the injection of syngeneic tumour cells as late as 1 day

before i.v. challenge inhibited the development of artificial metastases. It had, however, a less consistent effect upon the cytostatic activity of peritoneal exudate cells harvested within 2 days of tumour injection.

MATERIALS AND METHODS

Mice.—Almost all the experiments were performed in inbred CBA/Ca mice (male and female) aged 8–12 weeks. The remaining experiments were undertaken either in F_1 hybrids of CBA/Ca and A/HeJ mice or inbred C3Hf/Bu mice. The CBA/Ca mice were bred from mice obtained from the MRC Laboratory Animals Centre, Carshalton, Surrey. The A/HeJ mice used in the breeding of the (CBA/Ca × A/HeJ) F_1 hybrids were purchased from the Jackson Laboratories, Bar Harbor, Maine, U.S.A. The C3Hf/Bu mice were derived from stock originally obtained from Baylor University, Texas, U.S.A. They were kindly provided by Dr W. H. McBride of the Department of Bacteriology, University of Edinburgh.

Tumours.—A number of tumours were used in these studies. They include (a) MC fibrosarcomas from a number of strains of mice; (b) a fibrosarcoma which had been obtained by injecting CBA/Ca mice with syngeneic embryo cells which had transformed *in vitro*; and (c) an adenocarcinoma which had spontaneously appeared in CBA/Ca mice. Further details on the origin, generation number and designation of these tumours are presented in Table I.

Cultured MC-induced CBA fibrosarcoma cells (CCH1) were generally used for pretreatment and always used for challenge. These cells had been maintained for 3–18 months in culture, as previously described (Ghaffar *et al.*, 1974). The T3 fibrosarcoma cell line was also maintained in culture. All the other tumour-cell suspensions used were obtained by pronase digestion of freshly excised tumour cells (Woodruff and Boak, 1966).

X-Irradiation.—Tumour cells were irradiated at a dose rate of 274 rad/min to a total of 22,000 rad using a Westinghouse X-ray machine operating at 220 kV and 15 mA, with HVL of 1·2 Cu under conditions of maximum backscatter.

In vivo *experimental model.*—The basic protocol involved injecting mice s.c. with 10^6 syngeneic or allogeneic tumour cells 1 to 2 days before i.v. challenge with 5×10^4 tumour cells. The mice were killed 14 days after challenge, the lungs removed and fixed in Bouin's solution and the number of artificial metastases (tumour nodules) per lung counted. Each experimental group contained 6 to 19 mice. Further details on variations in this basic protocol are reported elsewhere in the text or in the footnotes to the figures and tables.

In vitro *cytostatic assay.*—The procedure used has been described in greater detail elsewhere (Ghaffar *et al.*, 1974). In essence it involved the addition of test effector cells to tumour-cell monolayers in the wells of plastic microculture plates and incubating the plates at 37°C for 48 h. At this stage, the culture medium (see below) was replaced by fresh medium containing ^{125}I-iododeoxyuridine (Radiochemical Centre, Amersham, England). The incorporation of this thymidine analogue into the tumour-cell monolayer was assessed 20 h later in an LKB Wallac gamma scintillometer. The spleen cells whose cytostatic activity was being assessed were obtained by gentle disruption in cold medium in a hand-operated glass homogenizer while the peritoneal exudate cells were obtained by washing out the peritoneal cavity with 3 ml of medium containing 10 u of heparin/ml. These cells were always washed ×3 before use. Throughout these *in vitro* studies, the medium used was RPMI 1640 (Gibco Biocult, Paisley, Scotland) buffered with 20 mM HEPES and supplemented with 10% (vol/vol) foetal calf serum, 100 u/ml penicillin and 100 μg/ml streptomycin.

Bacteriological and virological investigations.—A variety of standard procedures were used to ascertain if the cultured CBA MC fibrosarcoma cell line routinely used in these studies (that is, CCH1) was contaminated with micro-organisms which might influence antitumour responses. In brief these included electronmicroscopic examination of tumour cell sections, the screening of cells and culture supernatants for mycoplasma (McKay *et al.*, 1974), reverse transcriptase assays using the method of Dr Natalie Teich (personal communication) and indirect immunofluorescence on acetone-fixed cells (Hart and Marmion, 1977) with antisera to Moloney leukaemia virus and RD-114, an endogenous feline RNA oncovirus. Finally,

the cells were grown in the presence of ^{3}H-uridine and the supernatants screened for the presence of micro-organisms containing RNA. Further details on the culture procedure and the density-gradient separation technique used in the incorporation studies are reported elsewhere (Norval and Marmion, 1976).

Presentation of results.—The number of artificial metastases observed in experimental mice have been presented in scattergram form and the significance of the results has been determined using the Wilcoxon Rank Sum test. The *in vitro* cytostatic results have been expressed as geometric means together with the limits of one standard error from the mean ct/min of 5 replicate samples. The cytostatic indices (CI) have been calculated using the following formula:

$$CI = \frac{(N - T) \times 100}{N}$$

where N represents the mean ct/min obtained using effector cells from untreated (control) mice, and T represents the mean ct/min using effector cells from mice treated with tumour cells. The significance of the cytostatic results has been assessed by the standard Student's two-tail t test.

RESULTS

The effect of the preinjection of tumour cells on the development of artificial lung metastases

In our initial experiments, mice were injected s.c. with 10^6 viable cultured tumour cells and challenged i.v. 24 h later with various doses of cultured syngeneic tumour cells. The number of artificially induced pulmonary metastases present 14 days later was determined. The results of one such experiment are presented in Fig. 1. It will be noted that the number of metastases was significantly reduced in mice which had been injected s.c. with syngeneic tumour cells 24 h before i.v. challenge.

These initial observations were unexpected in view of previous suggestions that such pretreatment might interfere with the antitumour activity of host macrophages (for example see North *et al.*, 1976*a*, *b*). We decided therefore to perform additional experiments to see whether the effect observed was dependent upon the dose and route of injection of the tumour cells, the time they were injected in relation to i.v. challenge, and whether or not a similar effect could be achieved with lethally irradiated syngeneic tumour cells or with other syngeneic and allogeneic tumours.

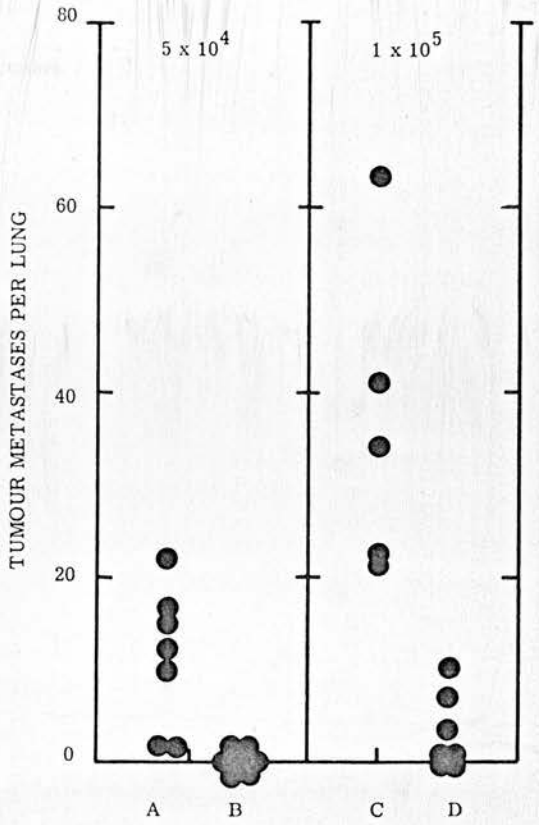

FIG. 1.—The effect on the growth of i.v. injected tumour cells of the preinjection (s.c.) of syngeneic tumour cells. A, C—no pretreatment; B, D—tumour cells s.c. Mice were pretreated by s.c. injection with 10^6 cultured syngeneic MC fibrosarcoma cells (CCH1) 24 h before i.v. challenge with 5×10^4 or 1×10^5 CCH1 tumour cells. Note that 14 days after i.v. challenge the number of lung metastases in the pretreated groups (B and D) was significantly lower ($P < 0{\cdot}01$) than in groups receiving no pretreatment (A and C).

Several experiments were performed to determine the dose dependency of the

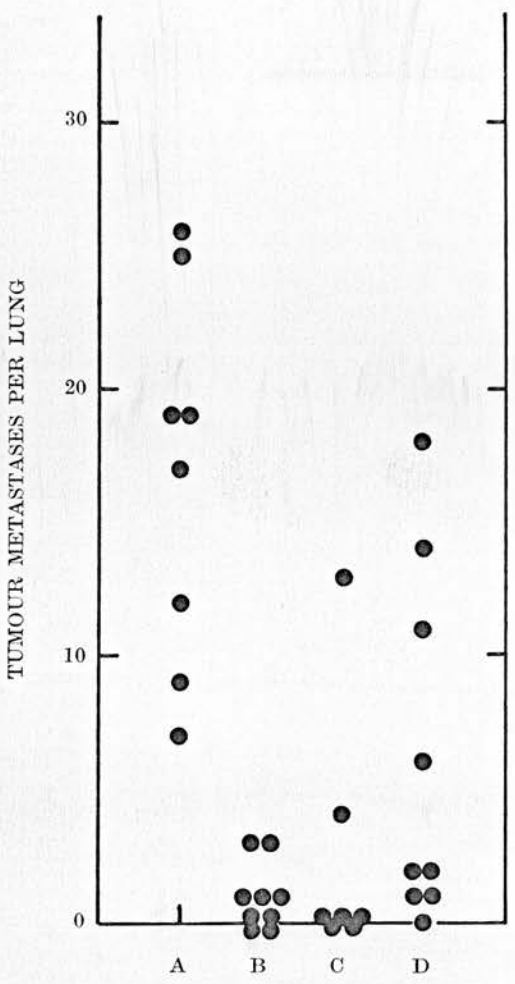

FIG. 2.—The effects on the growth of i.v. injected tumour cells of the preinjection (s.c.) of various doses of syngeneic tumour cells. Mice in Groups B, C and D were injected respectively s.c. with 10^7, 10^6 and 10^4 cultured syngeneic MC fibrosarcoma cells (CCH1) 24 h before i.v. challenge with 5×10^4 CCH1 tumour cells. Group A mice had no pretreatment. Note that 14 days after i.v. challenge the number of metastases in animals pretreated with 10^7 (B) and 10^6 (C) tumour cells was significantly lower ($P<0.01$) than in mice receiving no pretreatment (A). The inhibition observed in animals pretreated with 10^4 tumour cells (D) was just significant at the 0.05 level.

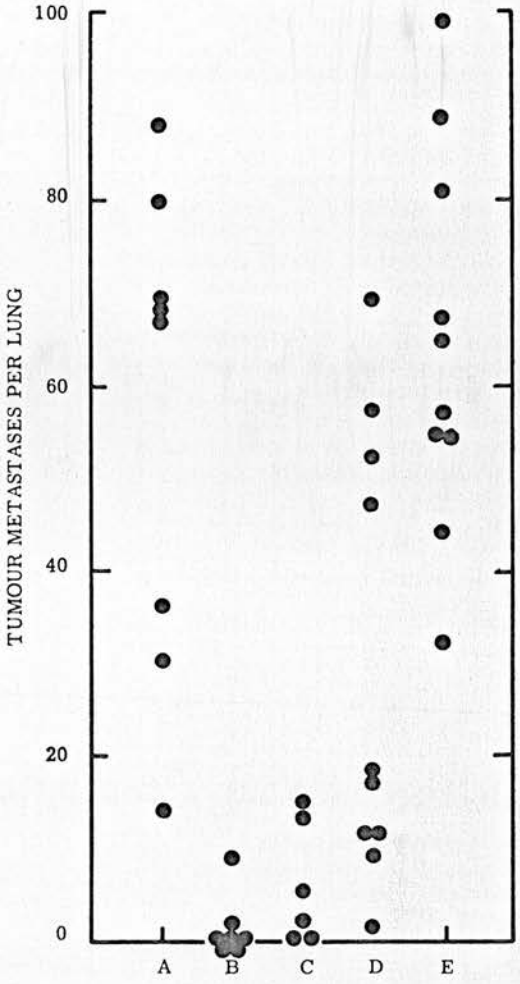

FIG. 3.—The effects on the growth of i.v. injected tumour cells of the preinjection (s.c.) of various tumour cell preparations. All mice received i.v. challenge with 5×10^4 cultured CCH1 fibrosarcoma cells. Pretreatment: A—no pretreatment; B—syngeneic MC fibrosarcoma cells Day -1; C—lethally irradiated syngeneic MC fibrosarcoma cells Day -1; D—syngeneic MC fibrosarcoma cells Day $+1$; E—allogeneic MC fibrosarcoma cells Day -1. Syngeneic MC fibrosarcoma cells were from cultured CCH1. Allogeneic MC fibrosarcoma cells were from freshly excised ACH3. Note that 14 days after challenge the number of tumour metastases per lung was significantly lower in mice pretreated with viable or lethally irradiated syngeneic tumour cells (B and C, $P<0.01$). In contrast, pretreatment with allogeneic tumour cells or the administration of tumour cells after i.v. challenge did not inhibit tumour metastases. (For further results of a similar nature see Figs. 5 and 7.)

effect, and the results of one of these experiments are presented in Fig. 2. These studies revealed that 10^6 tumour cells or

more had to be injected s.c. to ensure effective inhibition metastasis.

Experiments were then undertaken to see whether the protection observed above could also be achieved by preinjection of X-irradiated syngeneic tumour cells or allogeneic MC fibrosarcoma cells of A/HeJ origin. These studies revealed that the preinjection of X-irradiated syngeneic tumour cells also inhibited the development of artificial metastases following i.v. challenge, whilst pretreatment with fibrosarcoma cells of A/HeJ origin (designated ACH3) was without effect. It should also be noted that syngeneic tumour cells failed to inhibit tumour growth if administered after i.v. challenge. This phenomenon was investigated further (see Fig. 5).

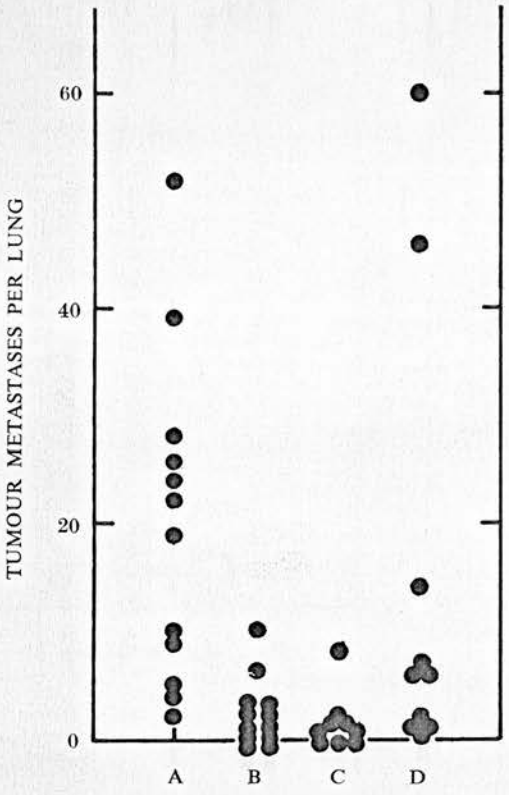

FIG. 4.—The effects on the growth of i.v. injected tumour cells of the preinjection of X-irradiated syngeneic tumour cells by different routes. Mice B–D were injected by the route indicated with 10^6 lethally irradiated cultured syngeneic MC fibrosarcoma cells (CCH1) and were challenged i.v. 2 days later with 5×10^4 CCH1 tumour cells. Note that 14 days after challenge the number of lung metastases in the s.c. (B) and i.p. (C) pretreated groups were significantly lower ($P<0{\cdot}01$) than in the control group (A), while those in the i.v. pretreated group (D) were not significantly different ($P<0{\cdot}01$).

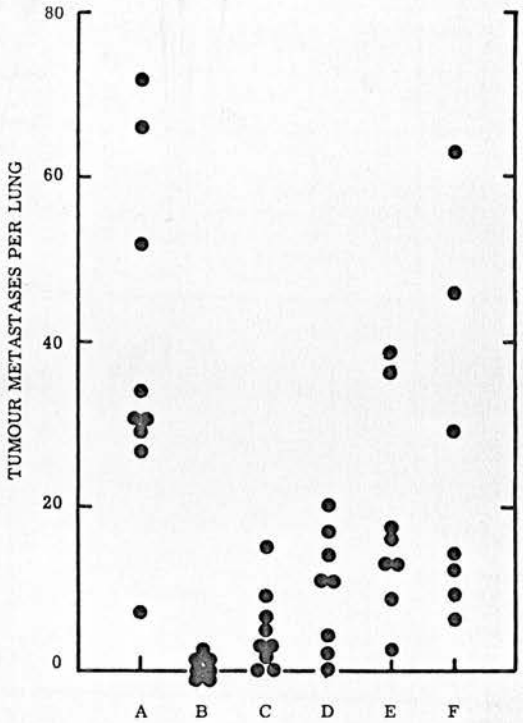

FIG. 5.—The effect on the growth of i.v. injected tumour cells of the s.c. injection of syngeneic tumour cells. Mice in Groups B–F were injected s.c. with 10^6 cultured syngeneic MC fibrosarcoma cells (CCH1) at various times (Day −3 to Day +1 respectively) in relation to challenge i.v. with 5×10^4 CCH1 tumour cells. Note that the number of tumour metastases 14 days after i.v. challenge was significantly reduced ($P<0{\cdot}01$) in the pretreated groups (B–D) but was not significantly affected in animals injected at the same time (E) or 24 h *after* i.v. challenge (F). See also Fig. 3, (D).

Investigations were then made into whether the protective effect afforded by preinjection of syngeneic tumour cells shortly before challenge was dependent upon the route of administration of the original inoculum. In this experiment

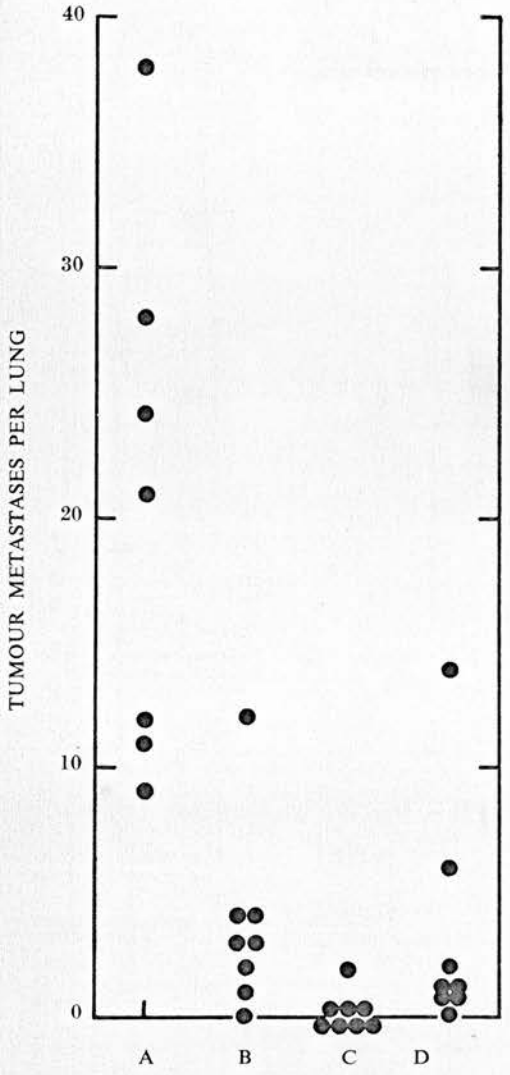

FIG. 6.—The effects on the growth of i.v. injected tumour cells of the preinjection (s.c.) of syngeneic tumour cells. Mice in Groups B–D were injected s.c. with 10^6 cultured syngeneic MC fibrosarcoma cells (CCH1) at various times (Days -14, -7 and -2 respectively) in relation to i.v. challenge with 5×10^4 CCH1 tumour cells. Note that the number of tumour metastases observed 14 days after i.v. challenge was significantly decreased in all pretreated groups ($P<0{\cdot}05$ to $P<0{\cdot}01$).

mice were injected s.c., i.p. or i.v. with 10^6 irradiated tumour cells. Significant inhibition of pulmonary metastasis was noted following preinjection by the s.c. and i.p. routes, but not by the i.v. route (see Fig. 4). This effect has been observed on 3 separate occasions.

The next 2 experiments were prompted by previous observations that the pre-

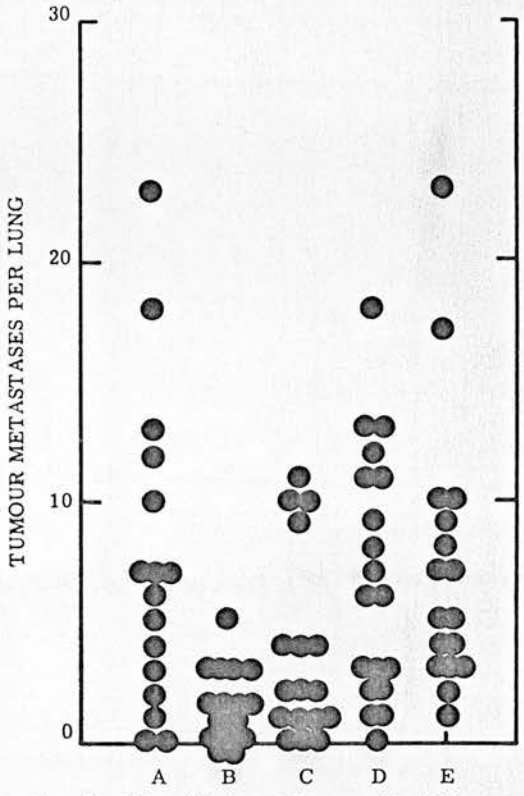

FIG. 7.—The effect on the growth of i.v. injected MC fibrosarcoma cells of the preinjection (s.c.) of various tumour cells. Mice were injected s.c. with 10^6 viable cells from a variety of freshly excised tumours and challenged i.v. 48 h later with 5×10^4 cultured MC fibrosarcoma cells (CCH1). Pretreatment: A—no pretreatment: B—syngeneic MC fibrosarcoma (CCH1); C—syngeneic spontaneous tumour (W54); D—allogeneic MC fibrosarcoma (ACH3); E—allogeneic MC fibrosarcoma (FSA). Note that 14 days after challenge the only significant reduction in lung metastasis was observed in mice pretreated with CCH1 tumour (B, $P<0{\cdot}01$). On this occasion the spontaneous tumour (W54, C) also inhibited tumour growth, though the effect was not significant, and in the 7 additional experiments performed with this tumour the inhibitory effects were much less marked.

TABLE I.—*A Summary of the Effects of Preinjection* (s.c.) of Various Tumours on the Growth in CBA Mice of a Syngeneic MC Fibrosarcoma (CCH1) Injected (i.v.) 24–48 h Later*

The tumours preinjected					Times inhibited/ times tested†
Description	Tumour designation	Generation Nos.	Strain of origin	How induced	
Fibrosarcoma	CCH1	18–19	CBA/Ca	With 3 methylcholanthrene‡ (Woodruff, Inchley and Dunbar, 1972)	20§/28
Fibrosarcoma	CCH5	1–2	CBA/Ca	With 3 methylcholanthrene (Woodruff *et al.*, 1972)	0/3
Fibrosarcoma	T3	¶	CBA/p	By injection of embryo cells spontaneously transformed *in vitro* (Smith and Scott, 1972)	0/1
Adenocarcinoma	W54	4–5	CBA/Ca	Spontaneous origin (Woodruff and Whitehead, 1977) (in preparation)	0/8
Fibrosarcoma	ACH3	26–27	A/HeJ	With 3 methylcholanthrene (Woodruff *et al.*, 1972)	0/8
Fibrosarcoma	FSA	6	C3Hf/Bu	With 3 methylcholanthrene (Suit and Suchato, 1967)	0/3

* Mice were injected s.c. with 10^6 viable tumour cells and 24–48 h later challenged i.v. with 5×10^4 viable CCH1 fibrosarcoma cells.

† The number of experiments in which significant inhibition ($P < 0{\cdot}05$) of lung metastases formation was observed out of the total number of experiments performed.

‡ This tumour is highly immunogenic, a single inoculum of 10^6 irradiated cells (22,000 rad) conferring complete protection to mice challenged 2 weeks later with 10^4 viable tumour cells (Woodruff and Dunbar, 1973).

§ In all 28 experiments the growth of tumour was inhibited, but this inhibition failed to reach significant levels in 8 experiments. On all these occasions a suspension of freshly excised tumour cells had been injected.

¶ Maintained in culture.

Note: Significant inhibition of growth of i.v.-injected cultured CCH1 tumour cells is only achieved after preinjection of CCH1 tumour cells.

ence of s.c. growing tumour reduced the number of artificial pulmonary metastases which could be induced by the i.v. injection of syngeneic tumour cells (Milas *et al.*, 1974). These workers also demonstrated by adoptive transfer that the resistance conferred was immunologically mediated, protection being most effectively transferred by cells from mice challenged 12 days previously with viable tumour cells. We decided therefore to investigate in more detail the development of artificial pulmonary metastases in mice which had been injected s.c. with syngeneic tumour cells at various times before and after i.v. challenge.

The initial "short-term" pretreatment experiment involved injecting mice s.c. with 10^6 viable syngeneic tumour cells on either Day -3, -2, -1, 0 or $+1$ in relation to i.v. challenge with 5×10^4 tumour cells, and 2 weeks after challenge the number of lung metastases formed was assessed. Significant suppression of metastasis was observed in all mice pretreated with syngeneic tumour cells, but was not achieved in mice treated after i.v. challenge (Fig. 5), thus confirming our preliminary observation (Fig. 3).

A further experiment was performed in which mice were injected s.c. with 10^6 viable syngeneic tumour cells 14, 7 and 2 days before i.v. challenge. A significant reduction in pulmonary metastasis was noted following pretreatment at all these times.

The specificity of the protection conferred was assessed by pretreating the CBA mice with a variety of tumours of syngeneic and allogeneic origin. The results of one such experiment are shown in Fig. 7. It will be observed that pretreat-

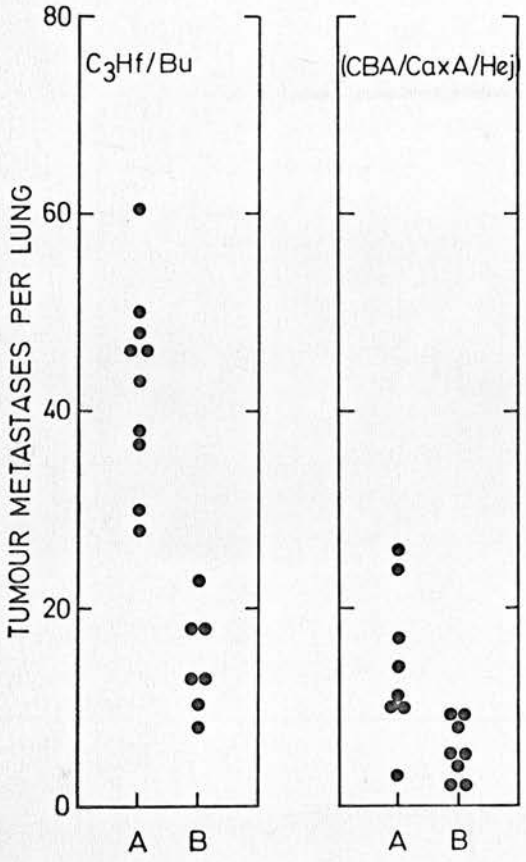

FIG. 8.—The effect on the growth of i.v. injected tumour cells of the preinjection of tumour cells s.c. In these experiments C3Hf/Bu mice were injected s.c. with 10^7 viable cultured syngeneic tumour cells (FSA) and challenged i.v. 1 day later with 10^5 of the same tumour cells. The (CBA/Ca × A/HeJ) F_1 hybrid mice on the other hand were pretreated with 10^6 cultured viable MC fibrosarcoma cells of CBA/Ca origin (CCH1) and challenged i.v. 1 day later with 5×10^4 CCH1 tumour cells. Note that 14 days after challenge the number of artificial metastases in the tumour pretreated groups (B) were significantly less ($P<0.01$) than in the untreated controls (A).

ment with only the syngeneic MC fibrosarcoma (CCH1) significantly inhibited the formation of artificial metastases following i.v. challenge with CCH1. The specificity of this phenomenon was further confirmed in a whole series of experiments, the results of which are summarized in Table I. It should be noted that to date we have failed (despite repeated testing) to significantly inhibit the growth of the CCH1 fibrosarcoma by pretreatment with other syngeneic and allogeneic MC fibrosarcomas and a syngeneic tumour of spontaneous origin.

In order to ensure that the phenomenon observed was not unique to the CBA/CCH1 combination, experiments of a similar nature were performed in C3H mice with syngeneic MC fibrosarcoma (FSA) and in (CBA/Ca × A/HeJ) F_1 hybrid mice using the CCH1 tumour. These experiments revealed (Fig. 8) that similar effects to those noted above could be obtained in other mouse tumour combinations.

A number of other interesting points emerged during the course of these studies: (a) similar protective effects could be achieved following pretreatment (in Day −2) with UV-irradiated cultured CCH1 tumour cells; (b) the tumours which developed in protected mice were much smaller than those observed in control mice; (c) tumour metastases were not observed in extrapleural sites; and (d) preliminary studies indicated that preinjection of cultured CCH1 tumour cells did not influence the clearance of ^{125}I-labelled PVP or ^{51}Cr-labelled CCH1 tumour cells injected one or 2 days later.

Finally, an overall analysis of the effects achieved by pretreatment with CCH1 tumour cells revealed that cultured tumour cells were much more effective than freshly excised tumour cells. Significant protection was observed in all experiments (17/17) involving pretreatment with cultured CCH1 tumour cells, but in only 3/11 experiments with freshly excised CCH1 cells.

The effect of preinjection of tumour cells on the in vitro *cytostatic activity of peritoneal and other cells*

In these studies mice were usually injected s.c. with 10^6 cultured, syngeneic MC-induced fibrosarcoma cells (CCH1) and 24 or 48 h later the cytostatic effect of their peritoneal exudate cells (PEC),

TABLE II.—*Effect of Preinjection (s.c.) of Untreated or X-irradiated Syngeneic Fibrosarcoma Cells (CCH1) on the Cytostatic Activity of Peritoneal Exudate Cells on CCH1 Target Cells*

Mouse treatment (Day −1)	Effector: target ratio	ct/min (Geometric mean of 5 replicates)*	P†
0·1 ml Saline s.c.	80:1	74,110 (69,924–78,546)	
	40:1	99,029 (90,502–108,360)	
	20:1	103,082 (100,612–105,613)	
10^6 Syngeneic MC fibrosarcoma cells s.c.	80:1	45,843 (37,658–55,807)	<0·05
	40:1	98,221 (97,415–99,034)	NS
	20:1	109,911 (102,725–117,599)	NS
10^6 X-irradiated (22 krad) syngeneic MC fibrosarcoma cells s.c.	80:1	59,977 (58,046–61,972)	<0·02
	40:1	97,017 (95,023–99,052)	NS
	20:1	93,890 (91,541–96,300)	<0·05
In vitro tumour control		98,093 (94,911–101,382)	

* Figures in parentheses represent mean ± s.e.

† 2-tailed Student's *t* test comparison of test groups with saline control. Values of $P>0·05$ were considered not significant (NS).

Note: Increased cytostasis following injection with non-irradiated and lethally irradiated tumour cells apparent with the 80:1 effector:target ratio.

TABLE III.—In vitro *Cytostatic Activity of PEC*: Effect of Removing Glass-adherent Population*

Mouse treatment (Day −2)	Effector: target ratio	ct/min (Geom. mean ± s.e.) Whole PEC	ct/min (Geom. mean ± s.e.) Non-adherent† PEC
0·1 ml saline s.c.	80:1	2,494 (2,368–2,627)	15,534 (14,699–16,416)
	40:1	21,486 (20,384–22,648)	27,901 (27,153–28,670)
10^6 Syngeneic MC fibrosarcoma cells (CCH1) s.c.	80:1	446 (353–562)	14,701 (13,514–15,991)
	40:1	14,447 (13,398–15,578)	28,291 (27,977–28,609)
In vitro tumour control		36,435 (33,945–39,109)	

* Peritoneal exudate cells.

† Adherent cells removed by incubating PEC in large tissue-culture flasks for 1 h.

Note: The marked reduction in the cytostatic effect of PEC on CCH1 tumour monolayers after removal of glass-adherent cells. This was apparent with PEC from both saline controls and tumour-pretreated mice.

spleen cells etc. on monolayers of the same tumour was assessed. In the majority of experiments, this short-term pretreatment produced a significant increase in the cytostatic activity of peritoneal exudate cells. Results from a typical experiment are presented in Table II. It was generally found that PEC from control mice express a cytostatic effect at the higher effector-to-target ratios and that preinjection with tumour increases this effect. It will also be observed that lethally irradiated tumour cells were also capable of stimulating the cytostatic activity of PEC. To date, no effect on the antitumour activity of cells from peripheral blood, spleen and lymph-node cells has been observed using this short-term tumour pretreatment (data not shown).

The cytostatic component of both normal PEC and tumour-stimulated PEC appeared to be associated with a glass-adherent population (Table III). This would suggest that the effects are mediated by macrophages but this will require confirmation by further analysis.

Further studies revealed that the increased cytostatic activity of PEC after injection with syngeneic tumour (CCH1) could also be achieved using an allogeneic

TABLE IV.—*Effect of Preinjection (s.c.) of Various Tumours on the* In vitro *Cytostatic Activity of PEC on CCH1 Tumour Monolayers*

Mouse treatment (Day −2)	Effector: target ratio	ct/min (Geom. mean of 6 replicates ± s.e.) Expt. 1	Expt. 2
0·1 ml Saline s.c.	80:1	15,472 (14,274–16,771)	345 (334–356)
	40:1	42,774 (41,859–43,710)	1,975 (1,769–2,206)
	20:1	39,941 (38,876–41,037)	12,888 (11,625–14,288)
10^6 Syngeneic MC fibrosarcoma cells (CCH1) s.c.	80:1	565 (502–637)*	866 (691–1,086)†
	40:1	15,874 (15,199–16,578)*	21,927 (20,390–23,580)†
	20:1	39,173 (38,788–39,562)	39,221 (38,245–40,222)†
10^6 Allogeneic MC fibrosarcoma cells (ACH3) s.c.	80:1	2,700 (2,305–3,163)*	434 (406–464)†
	40:1	33,934 (33,252–34,631)*	5,732 (4,882–6,731)†
	20:1	38,715 (38,240–39,195)	36,195 (34,183–38,326)†
10^6 Syngeneic spontaneous adenocarcinoma cells (W54) s.c.	80:1	33,420 (32,753–34,100)†	3,055 (2,684–3,503)†
	40:1	38,036 (37,515–38,564)*	28,082 (27,041–29,163)†
	20:1	38,109 (37,401–38,830)	41,195 (39,563–42,894)†
In vitro tumour control		44,032 (43,502–44,570)	41,543

Notes: Expt. 1.—Treatment with both the syngeneic and allogeneic fibrosarcomas results in elevation of cytostasis at the higher effector:target ratios. Treatment with spontaneous tumour abrogated the cytostatic activity of PEC.

Expt. 2.—Marked anti-tumour effect of the saline control PEC. All tumour pre-treatments reduced this effect, particularly noticeable at the effector:target ratios of 40:1 and 20:1. As in Expt. 1, the spontaneous tumour was most effective in reducing the cytostatic activity of PEC.

Statistical significance: All tumour-treated groups compared with saline controls using a 2-tailed Student's *t* test.

* Significantly lower ($P<0·01$) than in control group.

† Significantly greater ($P<0·02$) than in control group.

MC fibrosarcoma of C3H origin (Expt. 1, Table IV). It was not observed, however, following pretreatment with a spontaneous syngeneic tumour (W54). On the contrary, treatment with the spontaneous tumour resulted in a marked decrease in cytostasis.

Abrogation or reduction of cytostasis has also been observed in a minority of experiments with the syngeneic and allogeneic MC fibrosarcomas (Expt. 2, Table IV) and appears to be associated with an extremely high cytostatic activity in the PEC of control mice. Such high levels of activity may be due to transient infection in the mouse colony, leading to stimulation of the lymphoreticular system as described by Hibbs, Lambert and Remington (1972) and Krahenbuhl and Remington (1974).

Finally, it should perhaps be noted that the marked cytotoxicity normally elicited in PEC after i.p. *C. parvum* was not affected by injecting syngeneic MC fibrosarcoma cells (s.c.) one day before *C. parvum* injection (data not shown).

Bacteriological and virological studies

Four separate cultures of CCH1 have been examined. Three of the preparations were routinely used in these studies for pretreatment and challenge and they have been maintained in culture for 3–18 months. The remaining sample had been cultured for only a week.

To date there has been no evidence of contamination with micro-organisms in any of these preparations. No bacteria, mycoplasmas, viruses or virus-like particles were observed on electronmicroscopy of ultra-thin sections of tumour cells, and no mycoplasmas or bacteria were isolated by culture techniques. Density gradient ultra-centrifugation of ^{3}H-uridine-labelled culture supernatants failed to reveal any radioactivity banding in the positions characteristic of bacteria, mycoplasmas (density 1·22) lactic dehydrogenase-elevat-

ing virus (density 1·17) or RNA oncovirus (density 1·16–1·18). Furthermore there was no evidence of reverse-transcriptase activity in tumour-cell-culture supernatants. Finally, immunofluorescence tests with antisera to MLV and RD-114 also proved negative.

DISCUSSION

The present experiments clearly demonstrate that the preinjection (s.c. or i.p.) of live or lethally irradiated CCH1 methylcholanthrene-induced tumour cells into CBA/Ca mice shortly before challenge i.v. with the same tumour severely impaired the development of artificially induced lung metastases. In addition, peritoneal exudate cells recovered from mice injected one or 2 days previously with such tumour cells frequently exhibited an elevated *in vitro* cytostatic effect on syngeneic MC fibrosarcoma cells. The effects of lung metastasis were not readily achieved when small doses of cells (*i.e.* $<10^5$) were used in the pretreatment, when they were administered i.v., or if they were injected the day following antigenic challenge. The experiments also suggest that the *in vivo* protective effects are probably specific, significant protection only being conferred when the mice were pretreated with the same tumour as used for challenge. Furthermore, similar protective effects were noted in other mouse-tumour combinations.

The present observations are similar in certain respects to those noted in less extensive studies from other laboratories (Milas *et al.*, 1974; Yuhas, Pazmino and Wagner, 1975; McBride, personal communication). They differ, however, from those recently reported by other investigators in other models (North *et al.*, 1976; Pike and Snyderman, 1976; Nelson and Nelson, 1977) and are difficult to reconcile with previous suggestions that the initial survival of tumours may be due to their ability to suppress macrophage-mediated surveillance mechanisms (North *et al.*, 1976*a*, *b*; Nelson and Nelson, 1977; Pike and Snyderman, 1976; Snyderman and Pike, 1976).

At the present time, the mechanism whereby the preinjection of tumour-cells can inhibit the growth of the same tumour injected i.v. only one or 2 days later remains to be established. However, observations in other tumour systems suggest a number of ways in which this rapid effect might be achieved. In the first place, the injection of various tumours has been found to result in the appearance within one day of suppressor T cells (Fujimoto, Greene and Sehon, 1976), whilst cytotoxic cell activity was apparent within 3 days of challenge (Schick and Berke, 1976). Others have also shown that the injection of tumours or tumour-cell extracts in rats induces within 2 days mitosis in sinus macrophages in the draining lymph nodes and a peripheral blood monocytosis (Carr, Price and Westby, 1976). Additional studies had revealed that the ability of tumour to produce monocytosis appears to be directly related to its antigenicity (Eccles, Bandlow and Alexander, 1976). Thus, in theory at least, the effect might be due to a rapidly induced increase in T-cell cytoxicity or macrophage activity. Alternatively, if the initial growth of tumours is dependent upon immunostimulation (see Prehn, 1976) it might be due to the rapid development of suppressor T cells. In addition, the possibility of a prompt effect of natural killer (NK) cell activity cannot be excluded.

The observation that pretreatment with cultured tumour cells results in more effective protection than similar pretreatment with freshly excised tumour cells is of interest. There are several possible, though yet untested, explanations for this difference. For example, the normal host response to i.v. challenge may have been adversely influenced by any lymphoreticular cells which might have been present in freshly excised tumour cell suspensions. Alternatively, the tumour cells may have undergone modification during culture and as a result become more effective at

stimulating host defence mechanisms. Such mechanisms might include the aquisition of new tumour-specific transplant antigens or foetal calf serum antigens from the culture medium.

Our extensive bacteriological and virological investigations, together with the fact that the effect can be achieved with UV-irradiated cells, leads us to conclude that our observations are probably not due to contamination with micro-organisms such as the LDH virus which is known to be present in many tumours (Riley, 1968) and has been found to modulate the immune response of the tumour-bearing host (Kamo, Patel and Friedman, 1976). In any case it is known that the LDH virus, which replicates in macrophages (Kamo *et al.*, 1976), does not usually survive for more than 10 days in culture. Indeed this property is exploited to remove the virus from infected tumours (Riley, 1968). Nevertheless, the possibility still remains that the effect is due to contamination with some as yet undetected virus such as the minute virus of mice which has recently been shown to be responsible for the immunosuppressive effects of mouse EL-4 lymphoma cells on the mixed lymphocyte reaction (Bonnard *et al.*, 1976).

The present results are of particular interest in view of the recent flourish of reports indicating that tumours and tumour-cell products may interfere with the development and activity of cells of the monocyte macrophage series (see introduction and James, 1977). These observations have led to the suggestion that tumours may initially become established by suppressing macrophage-mediated surveillance mechanisms (North *et al.*, 1976*a*, *b*; Snyderman and Pike, 1976; Pike and Snyderman, 1976; Nelson and Nelson, 1977). The present observations, however, emphasize that the picture is far more complicated than suggested above, for pretreatment with tumour may on occasions inhibit rather than promote the growth of tumour injected shortly afterwards. In addition there is also a number of reports indicating that tumours may actually stimulate macrophage development and activity, these effects being noted within 2–3 days of tumour challenge (James, 1977). It is obvious that the effects of tumour on macrophage function are complex and diverse. Indeed it has recently been shown that short-term pretreatment with tumour may inhibit the chemotactic responses of peritoneal macrophages whilst simultaneously enhancing their capacity to phagocytose antibody-coated sheep erythrocytes (Meltzer and Stevenson, 1977*a*). Furthermore, it is apparent from the present studies that pretreatment with allogeneic tumour may enhance the cytostatic effect of peritoneal exudate cells without suppressing the development of tumour metastases. It is apparent, therefore, that further studies will be necessary to (a) explain these divergent effects; (b) elucidate their cellular and molecular basis; (c) establish their relative importance with respect to the initial phases of tumour growth; and (d) to identify and characterize the tumour products which modulate the host response to tumours. Finally it must be emphasized that such studies should be performed with tumours which are free (as far as can be reasonably ascertained) from contamination with viruses and other micro-organisms which are known to influence the function of cells of the lymphoreticular system.

The authors wish to thank Professor M. F. A. Woodruff, Dr W. H. McBride and Dr M. W. Scott for supplying the tumours used in these studies. They are also grateful to Mr J. Merriman for providing tumour cell cultures and to Mrs H. Hart and Mrs A. Graham, who respectively performed the immunofluorescence and ultrastructure studies. Finally they are indebted to the Cancer Research Campaign for their financial support.

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Normal human tissues contain RNA and antigens related to infectious adenovirus type 2

K. W. Jones, J. Kinross & N. Maitland

Institute of Animal Genetics, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh, UK

M. Norval

Department of Bacteriology, Medical School, University of Edinburgh, Edinburgh, UK

Normal human tissues have been found to contain RNA which hybridises with four regions of the adenovirus type 2 genome, including one which contains the transforming gene(s) of this virus. The RNAs have also been extracted from gorilla organs, but not from those of chickens, suggesting that they are a feature of all normal higher primate tissues. Serological tests suggest that these homologous RNAs are translated into proteins connected with essentially normal and virus-infected cell functions.

THE adenoviruses[1,2] are associated with respiratory infections in humans. Many serotypes of these viruses can induce tumours in rodents[3–6], and adenovirus type 5 transforms human cells in culture[7]. During studies on human cancer, we have investigated the possibility that human tissues might contain RNA sequences related to human DNA viruses, using the technique of *in situ* hybridisation[8–10]. We have found that normal human tissues contain RNA that hybridises with four regions of the genome of adenovirus type 2 (Ad-2). One of these regions includes the transforming gene(s) of this virus[7,11,12]. This suggests either that genes similar to viral transformation genes are present in man in

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much the same way that the avian *sarc* genes are homologous in base sequence to the *src* (transforming) gene of the Rous sarcoma virus[13,14], or that infectiously acquired Ad-2 DNA can persist under host control. The human cells expressing the RNAs in greatest abundance seem to be solitary, possibly circulating cells. Human tissue cultured cells apparently do not contain an abundance of these RNAs, which may account for the fact that they have not been described previously.

Hybridisation studies

In looking for possible RNA sequences related to Ad-2 viral DNA in uninfected human organs, we initially used placenta as the RNA source because it is available in quantity. Moreover, fetal tissues are often associated with endogenous viral sequence expression[15–17]. Total RNA was extracted by standard procedures[18] and iodinated[19] with ^{125}I for use in hybridisation. Equal amounts of Ad-2, human DNA and λ phage DNA or Ad-12 DNA were denatured and spotted on nitrocellulose strips and hybridised by the usual procedure[20] (for details see legend to Fig. 1). *Escherichia coli* total ^{125}I-labelled RNA served as an RNA control, hybridised to a parallel series of spots but substituting *E. coli* DNA for phage DNA. The hybridised strips were then treated with RNase and, after suitable washing, were dried and autoradiographed on presensitised X-ray film (Royal X-omat XHI Kodak) at −70 °C in cassettes fitted with intensifying screens. Such spot tests are extremely sensitive and the amount of signal due to hybridisation or to background is immediately visually apparent. Hybridisation can be quantitated if necessary by cutting out the spots and scintillation counting. This procedure was followed as described later to construct melting-temperature curves of the hybrids.

After autoradiographic exposure, there was no trace of hybridisation to phage DNA. In fact, the spots containing this DNA were, if anything, below background radioactivity levels in some cases, suggesting that the bound DNA may have pre-empted potential binding sites for molecules that contribute background. There was, as expected, very strong hybridisation to human DNA. Ad-2 DNA showed moderate hybridisation (Fig. 1*a*), which easily reached autoradiographic saturation on prolonged exposure (Fig. 1*b*). Ad-12 DNA showed a barely detectable autoradiograph (not shown). *E. coli* ^{125}I-labelled RNA did not hybridise with any of these DNAs, but did so with *E. coli* DNA.

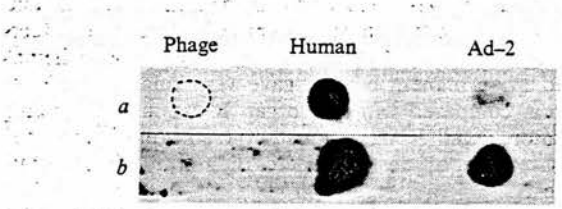

Fig. 1 Spot tests of hybridisation of ^{125}I-labelled human placental RNA to DNA of λ phage, human and adenovirus type 2 (Ad-2). The location of the negative hybridisation is shown by a dotted circle. Hybridisation has occurred to human DNA and to a lesser, but definite, extent to Ad-2 DNA. The conditions for hybridisation were as follows. The DNA samples were brought to the same concentrations, and 1-μg aliquots bound in spots to a strip of nitrocellulose filter by the method of Gillespie and Spiegelman[20]. The filter strip was then incubated for 6 h at 68 °C in 6×SSC containing 0.02% each of Ficoll, bovine serum albumin (BSA) and polyvinyl pyrollidone (PVP). It was then transferred to 3.6 ml of this same solution containing 2 mM, EDTA, 0.5% SDS and ~7×10^7 c.p.m. of placental total RNA previously prepared[18] and radiolabelled[19] with ^{125}I. The mixture was annealed at 68 °C for 15–16 h, cooled rapidly and the filter strip washed in 2×SSC, treated with RNase (40 μg ml^{-1}, 1 h, 37 °C in 2×SSC), washed in 2×SSC, treated with pronase (400 μg ml^{-1} in 2×SSC, 1 h), incubated in 2×SSC, 0.5% SDS for 6 h at 68 °C, washed exhaustively in a large volume of 2×SSC, dried in a vacuum oven and autoradiographed for (*a*) 10 d, (*b*) 3 weeks at −70 °C using Kodak Royal X-omat XHI X-ray film presensitised by brief exposure to light from a calibrated masked flash gun.

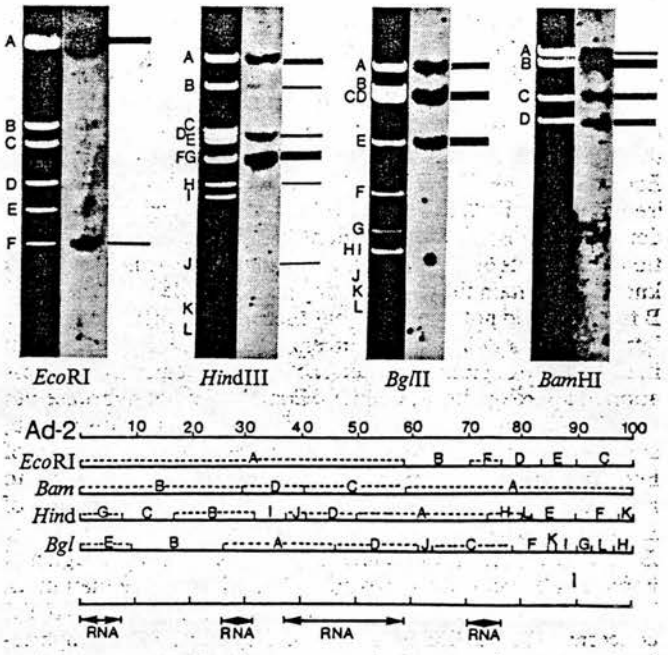

Fig. 2 Restriction digests of Ad-2 separated into fragment classes by electrophoresis on agarose gels stained with ethidium bromide and photographed in ultraviolet light, are shown paired with autoradiographs of ^{125}I-labelled placental RNA hybridised to the same fragments after transfer to nitrocellulose filter membrane and hybridisation as detailed in the legend to Fig. 1, and in the text. The bands in the digests are identified by letters to the left of each gel track. For technical reasons, the smallest restriction fragments do not photograph satisfactorily, and some of the minor autoradiographic bands are difficult to detect consistently together in the same autoradiograph. Because of this we have included a third track which indicates, diagrammatically, for each enzyme digest, all the bands to which we have obtained hybridisation in replicate experiments. These data are also shown below the photographs in the form of maps of the positions of the restriction fragments obtained with each enzyme in relation to the Ad-2 DNA map, which is divided into 100 fractions, according to the usual convention. The restriction fragments which hybridise with placental RNA are indicated for each digest by dotted lines over the fragment concerned. From these data, the provisional assignments have been made for the maximum limits to the Ad-2 genome sequences which hybridise with human total RNA; these are represented as double-ended arrows below the relevant locations. The conditions for electrophoresis were 1% agarose gels run at 40 V for 10 h, or 80 V for 4 h, in a buffer containing 15 mM Tris, 18 mM NaH_2PO_4, 0.5 mM EDTA, *p*H 7.8.

These results indicated that Ad-2 DNA contained some sequences homologous with human RNA, although they did not show whether these might have arisen from possible contaminating host cell sequences. Nevertheless, the fact that Ad-2 and Ad-12 were grown in similar human cells but behaved differently on hybridisation with human RNA suggested that contamination was unlikely. To investigate this point, we hybridised the same DNAs after digestion with the restriction endonuclease *Eco*RI, which cuts λ and adenovirus DNAs into small numbers of fragments; these can then be separated on agarose gels and transferred to nitrocellulose strips by the Southern procedure[21] for subsequent hybridisation with the same ^{125}I-labelled human RNA probe. After autoradiographic exposure there was hybridisation only to Ad-2 DNA and this was limited to the *Eco*RI A and F fragments (Fig. 2). Separate lots of Ad-2 DNA were then cut with three further restriction endonucleases, *Bam*HI, *Bgl*II and *Hind*III, which are known to produce genomal fragments overlapping and different from those produced by *Eco*RI. Hybridisation of human placental ^{125}I-RNA to these sets of fragments, as before, yielded the following hybridisation data (Fig. 2).

The fragments that hybridised were *Eco*RI A and F; *Hin*dIII A, B, D, FG, J and H (the latter two very faintly); *Bgl*II A, CD and E; and *Bam*HI A, B, C and D. These data define the following provisional maximum limits for the hybridising regions. As only the A and F fragments of *Eco*RI hybridised, all the observed hybridisation must lie within these limits. As *Eco*RI A, *Bam*HI B, *Hin*dIII G and *Bgl*II E fragments hybridised, but *Hin*dIII C or *Bgl*II B did not, the left end of *Hin*dIII C defines the most rightward limit of the first region of hybridisation at 7.5 units on the Ad-2 map[22]. This region, from 0 to 7.5, is known to contain the transforming gene(s)[23]. Because the *Bgl*II B fragment did not hybridise, but the *Bgl*II A fragment did, the latter, with the other restriction enzyme fragments that overlap it, defines the beginning (left end) of the next region of hybridisation at approximately 26 units. *Hin*dIII I did not hybridise but *Hin*dIII J did, so the I fragment defines a non-hybridising gap, and the left end of J defines the beginning of the next region of hybridisation at approximately 37 map units, extending to approximately 58.5 units, which is defined by the left end of *Eco*RI B, which did not hybridise. By the same reasoning the *Eco*RI F fragment must define the limits of the fourth region of homology as between approximately 70.7 and 75.9 map units. From this analysis, we presume that the doublet *Hin*dIII FG concerns hybridisation only to the G fragment, but that hybridisation occurs to both fragments of the *Bgl*II CD doublet. This point can be finally decided when a more complete analysis of the hybridisation patterns has been carried out. This provisional map summarising the maximum limits within which the hybridising regions lie is given diagrammatically in Fig. 2.

Fig. 3 Autoradiographs showing concentrations of Ad-2 complementary RNA in placenta. Ad-2 DNA labelled by the 'nick-translation' reaction with ^{3}H, was denatured and hybridised *in situ* to frozen sectioned human placenta in conditions that detect complementary cellular RNA[8–10]. *a*, A 'solitary' cell that shows a high concentration of label signifying a high concentration of Ad-2-related RNA. This cell type occurs relatively infrequently (about 2–3 per ×40 objective field) and occasionally seems to be loosely attached to the tissue (*b*). This may reflect the fact that it is a circulating cell. *c*, An example of a generally labelled area of placenta, after hybridisation as above. The grain distribution in this autoradiograph suggests that whole areas of this tissue contain Ad-2-related RNA at a lower level than the solitary, highly labelled, cells shown in *a* and *b*. Autoradiographic exposure 3 weeks. Controls of similarly hybridised λ phage DNA were negative. Scale bars, 10 μm.

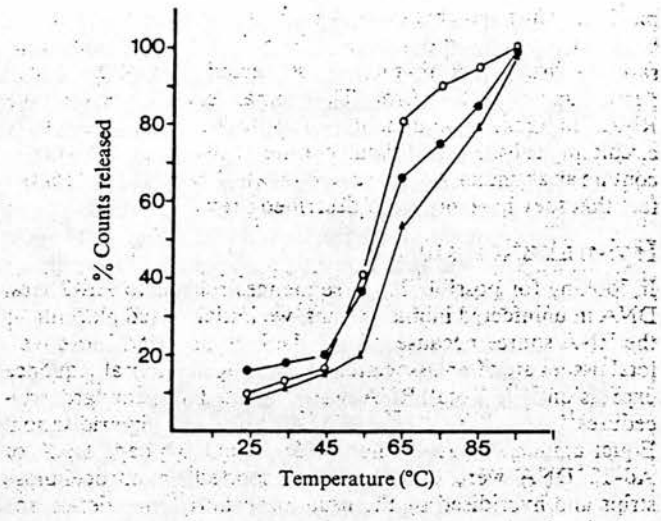

Fig. 4 Thermal dissociation of hybrids formed between human total ^{125}I-labelled RNA and human total DNA (○), and Ad-2 whole genomal DNA (●, sample 1; ▲, sample 2), from different sources. The curves show similar features, with both Ad-2 DNA samples having formed hybrids of rather higher thermal stability than total human DNA. Hybridisation conditions are described in the legend to Fig. 1. Dissociation was carried out by cutting spots of hybridised DNA from nitrocellulose filters of spot tests that had been shown to be positive by previous autoradiography on X-ray film as in Fig. 1. These were then counted by scintillation spectrometry in toluene-based scintillation fluid, washed and then treated in diethyl pyrocarbonate 0.1 in SSC (0.15 M NaCl, 0.015 M sodium citrate, *p*H 7.4) for 30 min, washed in SSC, and heated for 10 min in 1 ml 0.1 SSC and 0.1% SDS at each of the temperatures shown. The counts released were recovered from solution by trichloroacetic acid precipitation in the presence of 100 μg BSA per sample, dried by suction filtration and counted by scintillation spectrometry. Approximately 80% of the original radioactivity was recovered.

This provisional map has been confirmed with a further two independently obtained samples of Ad-2 DNA and with 10 separate samples of placentas, obtained from normal births, from two hospitals. We have also found the same pattern in the case of human adult liver RNA, and with liver, kidney, brain and spleen RNA obtained from a gorilla which died from the effects of minor surgery. The RNAs from four separate human cell lines maintained in permanent culture gave very faint patterns of hybridisation compared with the organ RNAs and are being studied in more detail. RNA obtained from chickens in the same manner as for other organs of primates did not hybridise in two separate tests.

That human organ RNA and other higher primate RNA contain sequences with homology to certain regions of the genome of Ad-2, but that such RNA apparently is relatively much less abundant in cell lines of human origin, indicated that the distribution of cells having such an abundance was not uniform. We investigated this aspect by hybridising ^{3}H nick-translated, denatured Ad-2 DNA to human placental cryostat sections *in situ* using a method which permits hybridisation to mRNAs[8–10]. After S_1 nuclease treatment and thermal rinsing to remove unhybridised probe or poorly matched hybrids, the preparations were autoradiographed and examined microscopically. Strong hybridisation occurred only to certain cells in the sections. These seemed to be solitary cells, with hybridisation to almost the entire area of the cell (Fig. 3*a*, *b*). Other areas of the sections showed a lower level of hybridisation which was, however, above the non-cellular background (Fig. 3*c*). There was no hybridisation in the case of λ phage ^{3}H-DNA hybridised in identical conditions. We presume from this that the bulk of the RNA that hybridised is contributed by a tissue cell subpopulation and that cells of this type are not among the cell lines that we examined.

Melting-temperature experiments

We carried out melting-temperature experiments to determine the thermal stability of the the hybrids formed on nitrocellulose between Ad-2 DNA and placental RNA. These were done by cutting out the relevant hybridised spots from the filter and heating them in solution at increasing temperature (see legend to Fig. 4) to measure the loss of hybridised RNA from the filters. Hybrids formed between human ^{125}I-labelled total RNA and two separate Ad-2 DNA samples and with total human DNA were compared. As can be seen from Fig. 4, the melts of Ad-2 hybrids showed similar characteristics and similar, though slightly higher, T_m values compared to the homologous hybrids, indicating that the hybrids to Ad-2 were reasonably thermally stable. This result was expected from the annealing conditions (legend to Fig. 1) which were quite stringent and essentially identical to those used in most published Ad-2 hybridisation studies (see discussion). We conclude that the hybrids that we detect between human RNA and Ad-2 DNA are at least as well matched as those in previous transcription studies of Ad-2. We are now investigating methods to determine the precise lengths of the hybridised regions. However, this is complicated by the fact that the organ RNA is to some extent degraded.

Immunological studies

Hybridisation between a viral DNA and human RNA from apparently uninfected tissues suggests similar protein coding functions in the sequences concerned. To test this hypothesis, rabbit cells infected with a clinical isolate of Ad-2 were used to raise antisera separately against the early-infected and late-infected cells (see legend to Fig. 5). The antisera were then reacted with six human cell lines (not shown) in an indirect immunofluorescence assay. All except one cell type, a human glioma (NG-118H) transformed by Rous sarcoma virus which was faintly positive, were completely negative. Controls of Ad-2-infected HEp 2 cells were brilliantly positive (Fig. 5*a*) as were transformed rat cells containing the extreme left-hand fragment of the Ad-2 genome[12] (not shown). Placental sections were also positive when treated with the anti-early antisera (Fig. 5*b*,*c*), but not with anti-late-infected cell antiserum. The fluorescence was concentrated on the surfaces of cells lining what seemed to be channels in the tissue as well as occurring diffusely in the cytoplasm of many areas. Some cells showed the nuclear fluorescence usually seen in transformed cells. However, this was rare and not intense. Of 10 placentas tested, 8 were undoubtedly positive in these tests and 2 were judged negative. We do not understand this variation, as all were positive in hybridisation tests for Ad-2-related RNA; however, it may be due to the fact that the antigens concerned are unstable and that the variations reflected the variable storage conditions of the tissues before we obtained them.

Antigens were then solubilised[24] from placental tissue and tested by gel diffusion in Ouchterlony plates against both early- and late-infected cell antisera, using controls of Ad-2-infected rabbit cell antigens (Fig. 6). There was no reaction with any dilution of uninfected cell antigens (not shown). Strong precipitin lines formed between the early-infected cell antisera and the antigens of both early- and late-infected cells, as expected, for both will contain the same early antigens, although presumably in different concentrations. Weaker but quite definite precipitin lines formed between this same antiserum and the wells containing placental antigens, and these showed partial identity with one of the lines originating from the early-infected cell antigen wells but no identity with late-infection antigens. The tests against anti-late-infected cell antisera were even more strongly positive against the infected cell antigens, particularly the late-infected cells. However, there were no visible precipitin lines between this antiserum and the wells containing placental antigens. Separate tests were then carried out in which a range of dilutions of the placental antigens were tested against each antiserum separately. No dilution used gave any precipitin lines with the late-infected cell antiserum (not shown). The early-infected cell antiserum, however, showed a single precipitin line that altered position between the antigen and antibody well depending on concentration. This shift is due to antigen–antibody concentration which affects the solubility and hence the mobility of the antigen–antibody complex. The fact that there was no precipitin line in the case of anti-late-infected cell antiserum suggests that the concentration of the relevant antibodies to early-infected cell antigens was very low.

We conclude from these preliminary immunological experiments that rabbit cells infected with Ad-2 contain certain antigens that are not present in uninfected rabbit, or human, cells in culture. We further conclude that some of the Ad-2-induced antigens are identical to tissue antigens present in most

Fig. 5 Indirect immunofluorescence tests for antigens on: *a*, Ad-2 early-infected human cells (HEp 2); *b*, *c*, normal human placenta cryostat sections, using antiserum raised against Ad-2 early-infected rabbit cells (Rk-13), at a dilution of 1/40 and sheep anti-rabbit IgG conjugated with fluorescein isothiocyanate (Wellcome). The rabbit cells were grown in medium 199 supplemented with 2% rabbit serum and the antisera raised in rabbits. Cells were infected for 20 h in the presence of 20 $\mu g\ ml^{-1}$ cytosine arabinoside. The resulting antiserum was negative for virus neutralising activity. Uninfected cell antiserum was also raised and was negative by immunofluorescence with these same subjects, and infected cell antiserum was negative with both uninfected HEp and RK-13 cells. The infecting adenovirus was obtained from a clinical isolate. Antiserum to late-infected cells was negative in similar tests. Fluorescence in the HEp cells is mainly on nuclear structures with some cytoplasmic fluorescence. Placental sections show stronger fluorescence in cytoplasmic regions, especially on what are apparently cells lining channels or vessels in the placenta, but nuclear fluorescence is also visible. These results, which demonstrate the presence of cross-reacting antigens between early-infected and apparently uninfected placenta, have also been obtained with all other tissues but not with cell lines tested (see text), and are confirmed by Ouchterlony gel diffusion (Fig. 6 and text).

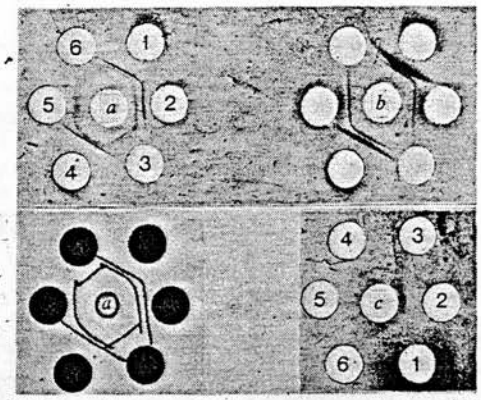

Fig. 6 Ouchterlony gels showing patterns of precipitation obtained between *a*, anti-early Ad-2-infected Rk-13 cells, and *b*, anti-late Ad-2-infected Rk-13 cell antisera. Wells are numbered 1–6 in the same manner in each case. Wells 1 and 4 contained dilutions of 1/50 and 1/100 of late Ad-2-infected antigens; 2 and 5 contained dilutions of 1/50 and 1/100 Ad-2 early infection antigens; 3 and 6 contained 1/50 and 1/100 dilutions of placental antigens. An interpretation of the pattern seen in *a* is shown below it. In *a*, precipitin lines can be seen between both wells containing placental antigens and the central well containing anti-Ad-2 early-infected Rk-13 cells. In 3 there is a suggestion of partial identity with one of the lines associated with well 2 as judged from the single spur. There are no lines visible between the central well and wells 3 and 6 in *b*, indicating absence from the late-infected cell of antigenic components related to those of normal placenta. Dilution experiments with this particular combination over the range of 1/10 to 1/1,000 of antigen concentration revealed no precipitation (not shown); however, a similar range of dilutions of the same combination in the case of *a* showed an arc of precipitation spiralling out from the centre well according to the dilution of antigen; this is shown in *c*, in which all wells contained placental antigen, as follows: (1) undiluted; (2) 1/10; (3) 1/50; (4) 1/100; (5) 1/1,000; (6) saline. These data confirm that normal human placentas express antigens that are associated with Ad-2 early infection as shown by immunofluorescence in Fig. 5.

human placenta samples, concerning mainly the early proteins of the adenovirus infectious cycle. Such antigens are not detectable on cell lines of human origin by immunofluorescence but were present in all tissues of gorilla (not shown). We are now attempting to characterise these antigens in more detail. The presence of antigens related to Ad-2 early function proteins in humans and in gorilla tissues correlates with the presence in these tissues of RNA molecules that hybridise with regions of the Ad-2 genome involved in coding early function proteins. We suppose from this that these RNAs code for the antigens that are shared between apparently normal human, and other primate, tissues. The *in situ* hybridisation result using Ad-2 DNA probes suggests that certain cells, or cells in a certain transitory state, which constitute a minor subpopulation in the placenta, contain a high concentration of the Ad-2-related RNA. The connection between this observation and the fact that the antigens concerned are rather ubiquitous in the tissues examined is unclear but is discussed below. However, as there are at least four regions of the Ad-2 genome that hybridise, it is possible that different cell types contain different proportions, or types, of the corresponding, homologous, RNAs, and consequently code for different antigenic moieties.

Discussion

The present findings are unique in the sense that there have been no previous reports of RNA sequences related to adenoviruses in apparently uninfected human cells. These findings are particularly intriguing as they relate to adenoviruses that are potent transforming viruses in animal hosts[3–6]. Moreover, the regions of homology include sequences in the extreme left-hand end of the Ad-2 genome that contains transforming genes of this virus. The consistency of the findings, there being no negative results in the 11 human tissue samples included in this study, indicates that the RNAs concerned may be ubiquitous in the population. Moreover, the presence of the same RNA molecules in gorilla is indicated from the similarity of the hybridisation patterns obtained with RNA from kidney, liver, spleen and brain. This suggests that the RNAs may also be common within the higher primate group, although more examples, involving some from a natural population, will be essential to support this.

Our criteria for hybridisation of the RNAs to Ad-2 DNA are those normally used in hybridisation mapping of Ad-2 (refs 25, 26) and are reasonably stringent taking the genome as a whole. Increasing the hybridisation temperature to 75°C reduced but did not prevent hybridisation. Moreover, the thermal stability of the Ad-2 DNA – human RNA hybrids seems representative of hybrids formed between human total DNA and the same human RNAs used in this work. We conclude from this that the hybrids are reasonably well matched. This is supported by the fact that there is demonstrable homology between the antigens coded during infection by Ad-2 and those present in human placenta and liver, and gorilla organs, which indicates similar coding functions of the RNA produced early in Ad-2 infection and that present in apparently normal higher primate organs.

These data, although consistent, are at present limited and the possible interpretations are, correspondingly, tentative. However, the findings are provocative and invite some speculations as to their significance. The RNAs concerned may have originated in either of two, not necessarily mutually exclusive, ways. Namely, host acquisition by an infectious process or the evolution of Ad-2 (and the C group adenoviruses) through the acquisition of nucleotide sequences similar to those found in the natural primate host.

The facts relevant to these interpretations are that only certain Ad-2 DNA regions are represented in human RNA. This tends to rule out a productive Ad-2 infection as the RNA source, as we would then expect to find RNA representative of the whole genome. Because all individuals investigated contained the RNAs in question, it is obvious that the mode of transmission is very efficient. This rules out a particular defective virus as the source unless we also assume that transmission is vertical and widespread. In this case, it is debatable whether such sequences belong to the host or to a virus. We cannot, however, exclude the possibility that the sequences we detect belong to a non-defective, incompletely Ad-2-related virus, in our view an unlikely explanation, or that Ad-2 DNA may be carried in the manner of Epstein–Barr virus[27], following infectious contact and subject to specific host transcriptional control.

The second hypothesis is that Ad-2 and related strains have evolved, or otherwise acquired, genes that are related to certain genes of their natural hosts. As it is known that Ad-5 can integrate with host DNA[28], such evolution seems likely. Also, RNA tumour viruses of chickens and mice contain sequences related to their host cells. In avian sarcoma viruses the transforming gene *src* is related in nucleotide sequence to the host gene *sarc*[13]. It is also known that the transforming region of a primate virus SV40, which is totally unrelated to Ad-2, is rather similar to Ad-2 in its transcriptional structure[29], although it is not known whether the proteins coded are functionally analogous. Such convergent evolution might reflect the fact that both viruses have acquired or evolved control elements concerned with the same host function, as both are primate viruses. Presumably, the advantage of such a mode of evolution would be that the virus would be better adapted to subvert host functions, for example, by using the host RNA splicing systems. Pressure to acquire control of the same system on the part of unrelated viruses could stem from the fact that the functions concerned are key elements in growth control. In this regard it is known that Ad-2 replicates in human cells significantly faster than Ad-12 (refs 30, 31), which does not, as far as we can detect, share the host-related nucleotide sequences.

Whether or not either hypothesis is wholly correct, our findings do indicate that human cells possess transcripts, from whatever source, closely related to DNA that maps within the

transforming region of the adenovirus genome. This may be relevant not only to how the virus itself replicates and transforms, but also to how malignant human cells may alter their growth patterns. Moreover, at least four regions of homology are detectable which may mean that at least four different RNAs related to the viral DNA exist in human cells. In future studies these can be recovered and their corresponding genes cloned and identified. In the event that the second hypothesis is substantially correct, such genes may relate to important functions connected with cell and virus growth control. A search for similar sequences related to other DNA viruses is also suggested from the fact that antigenic similarity has been described between SV40-induced surface antigens and fetal antigens in hamsters[32].

The fact that Ad-2 early function antigens cross-react immunologically with apparently ubiquitous antigens present in the natural host, regardless of their origin, is likely to be medically significant. Bacterial pathogens that share antigens with human cells can precipitate autoimmune reactions, as in the rheumatic disease caused by *Streptococcus pyogenes* infections. Persistent adenovirus infections might therefore, by analogy, precipitate similar diseases. Thus, there might be a link between other rheumatic conditions or autoimmune diseases and the antigens that we detect. Of particular interest here is the identity of the cell type that seems to synthesise a high concentration of Ad-2-related RNA as determined by *in situ* hybridisation. If such synthesis implies a high content of related antigens, such a cell might be particularly at risk from an autoimmune reaction directed at these common antigens.

The cellular source of the cross-reacting antigens in placental tissue is unclear. By immunofluorescence, these antigens are widely distributed in the tissue. However, they do not appear on tissue-cultured cells of human origin. Moreover, the cell type that apparently possesses the greatest amount of Ad-2-related RNA as determined from *in situ* hybridisation, and thus is presumably most likely to synthesise most antigen, is relatively rare. One possible explanation is that different RNA molecules, related to different regions of the Ad-2 genome, are not equally abundant in all cells. Another is that the antigens or their precursors circulate in blood or other body fluids and/or that their distribution is altered as a result of post-mortem changes, such as blood clotting. Such a mechanism would partly explain why these antigens have not generally been found on cells in culture.

We thank Joe Bonventre and Ashley Dunn for samples of adenovirus type 2, Phil Gallimore for rat Ad-2-transformed cell lines and Ad-2 virus samples, Professor B. Marmion for discussions, Jeanette Muir for technical assistance, and Dr Bruce Hobson and staff at the Royal Infirmary, and the staff of the Elsie Ingles Hospital, Edinburgh, for providing placental samples. This work was supported by the Cancer Research Campaign and the Nuffield Foundation.

Received 7 August; accepted 11 December 1978.

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Br. J. exp. Path. (1981) **62**, 270

GROWTH IN CULTURE OF ADENOCARCINOMA CELLS FROM THE SMALL INTESTINE OF SHEEP

M. NORVAL*, K. W. HEAD†, R. W. ELSE†, H. HART* AND W. A. NEILL*

*From the *Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh, and the †Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh*

Received for publication February 6, 1981

Summary.—Explant cultures were initiated from adenocarcinoma of the small intestine in sheep and from various metastases. Several cell types grew, most being fibroblastic in nature. However, 2 cultures yielded mixed cells which arranged themselves into areas of epithelial-like cells surrounded by fibroblast-like cells and this pattern was consistent over 30 subcultures and several months of culturing. The epithelial-like cells were separated from the others by the use of a modified medium containing citrulline or by sedimentation through a bovine serum albumin solution. Various properties, including their growth rate in 5% and 0·5% serum, the absence of surface fibronectin and their ability to grow in semi-solid agar, indicated that they may represent carcinoma cells. Screening for virus production from these cells and all other explant cultures proved negative.

ADENOCARCINOMA of the small intestine in sheep has been reported particularly in older ewes in New Zealand (Simpson and Jolly, 1974), Australia (Ross, 1980), Iceland (Georgsson and Vigfusson, 1973), Scotland (Norval, personal communication) and Northern England (McCrea and Head, 1978). In those parts of the world at least this tumour is found relatively frequently, but it should be noted that prevalence in abattoir surveys is controlled not only by the number of tumours in the sheep population of the district but also by the age and type of sheep sent for slaughter. Thus, in animals of about 1 year old and older (lambs and ewes) it is given as 0·97% in Iceland (Georgsson and Vigfusson, 1973) and 0·2% in Scotland (Norval, personal communication). Simpson (1972*a*) reported a range of from 0·2% to 1·58% of ewes slaughtered in 10 slaughterhouses in 7 geographic areas of New Zealand. Abattoir prevalence figures in New South Wales, Australia, ranged from 0·093% to 0·487% for adult sheep (Ross, 1980). Because there are difficulties in ensuring that the abattoir samples are comparable it is not certain if there is a geographical distribution in incidence (Head, 1967). The aetiology of the disease is not known. Simpson (1972*b*) demonstrated 16 factors were statistically associated with the tumour in New Zealand. It has been suggested that exposure to carcinogens in fodder (Georgsson and Vigfusson, 1973) or in bracken, *Pteridium aquilinum* (Evans and Mason, 1965) may be important but other factors, in particular viruses, may be involved. In this context, the recent work by Jarret *et al.* (1978) is of interest where a correlation has been drawn between bovine papilloma virus, an environmental carcinogen thought to come from bracken and alimentary carcinoma in cattle.

Ovine adenocarcinoma is found at any site along the length of the jejunum and ileum, but is not found in the duodenum. In sheep from this country, it is usually present as a solitary primary lesion. The cell type involved is thought to be either adsorptive cells or goblet cells (Simpson and Jolly, 1974). Tumours may spead from the intestinal site to the mesenteric

lymph node by lymphatic permeation, and there may be transcoelomic metastasis leading to lesions on the surface of many organs such as the peritoneum of the anteroventral flanks, diaphragm and spleen. In the final stages, a large volume of ascitic fluid is present.

This study was undertaken to examine the cells which grew *in vitro* from the primary adenocarcinoma tissue and from various metastases, especially to look at their morphology, ultrastructure and whether they were infected with viruses. One cell line of particular interest was obtained, ST–6, which produced a mixture of epithelial-like and other cell types on primary culture. The epithelial-like cells were separated from the others using sedimentation in bovine serum albumin solution and growth in citrulline-containing medium, and their characteristics examined in detail.

MATERIALS AND METHODS

Tumour material

Tissues from 11 sheep suspected by the meat inspectors of being examples of adenocarcinoma of the small intestine were obtained from the abattoir in Edinburgh. As soon as possible after slaughter, the primary lesion and/or any metastases were used for culturing purposes. The specimens were also the subject of morbid anatomical examination. Tissues were processed to paraffin wax, cut at 5 μm and stained with haematoxylin and eosin; in addition, where appropriate, Gordon and Sweet's stain for reticulin fibres, Alcian Blue and P.A.S. for mucin were also used.

Culture methods

(a) *Primary culture of tumour cells.*—The tumour specimen was cut into small pieces approximately 1 mm in size and several placed beneath a glass cover-slip in a plastic Petri dish (Sterilin). The medium used was Earles-based Eagles' complete medium containing 200 iu/ml penicillin, 200 μg/ml streptomycin, 50 iu/ml mycostatin and 100 μg/ml gentamicin, and supplemented with 10% newborn calf serum. All culturing was performed at 37° in 5% CO_2. Cells were subcultured using trypsin–versene. After subculture, the gentamicin and mycostatin were omitted from the medium and the serum content reduced to 5%. The cells were tested at intervals for the presence of mycoplasmas as described by Mackay *et al.* (1974).

(b) *Citrullin medium.*—ST-6 cells were cultured in Sun's modified Waymouth MB 752/1 medium containing either arginine or citrulline and supplemented with dialysed newborn calf serum (Sun *et al.*, 1979).

(c) *Separation of cell types using sedimentation in bovine serum albumin (BSA).*—The method described by Moore and McBride (1980) was used whereby cell suspensions were fractionated by velocity sedimentation at unit gravity. Briefly, 10^7 cells were sedimented for 90 min through a 1–2% BSA gradient in Dulbecco's solution contained in an 18cm diameter "Staput" chamber (Johns Scientific, Toronto).

(d) *Rate of growth.*—The rate of growth of ST-6 cells and its subpopulations was measured by putting 2×10^5 cells into 50mm Petri dishes in 5 ml Eagles' medium containing either 5% or 0·5% newborn calf serum, and counting the number of viable cells present, after removal with trypsin–versene, on each day for a period of incubation of 8 days.

(e) *Growth in semi-solid agar.*—The epithelial cells derived from the ST-6 culture were cloned in Eagles' medium with 10% newborn calf serum and containing 0·275% agarose (LGT pure agarose powder, 49-056, Miles Laboratories), with 0·55% underlay containing 10^5 human embryo lung cells/ml as feeder cells. The sheep cells were seeded at densities of 5×10^4 and 10^4 in 50mm Petri dishes.

Fibronectin immunofluorescence

An indirect immunofluorescence test to detect fibronectin was carried out as described by Chen, Gallimore and McDougall (1976), using cells grown in monolayers and fixed with 2% paraformaldehyde. Rabbit antiserum, specifically cross-reacting with mouse, rat and human fibronectin (data not shown), was produced by injecting purified human cold insoluble globulin into rabbits, and was kindly donated by Dr J. Kinross. It was used at a dilution of 1/40, while the FITC anti-rabbit conjugate was used at dilutions of 1/8–1/16.

Ultrastructure

Cells grown on plastic and glass flasks were harvested by gentle mechanical scraping using a rubber policeman or by enzyme digestion using neutral protease (Dispase II, Boehringer).

Some samples were fixed for 1 h in 2·5% glutaraldehyde before removal from flasks and others were fixed as cell suspensions after removal. After removal and fixation, cell pellets were formed in polyethylene conical-tipped tubes (Azlon) by centrifuging at 3000 *g* for 10 min. Pellets were washed twice in 0·1M sodium cacodylate buffer, post-fixed in 2% osmium tetroxide and then embedded in Spurr resin (EMscope Laboratories) before sectioning on an LKB Ultratome III microtome. Sections stained

with lead citrate and uranyl acetate were examined in a Phillips 400 TEM at an accelerating voltage of 100 Kv.

For scanning electron microscopy cells were cultured on 10mm diameter glass cover-slips in Petri dishes. When confluent, monolayers were fixed in 2·5% glutaraldehyde and prepared for scanning electron microscopy by critical-point drying using a Polaron critical-point drying apparatus. Samples were examined in a Cambridge Stereoscan 180 SEM.

Chromosome analysis

Sixteen hours after subculture into 25cm² flasks from a confluent monolayer, ST-6 cells were pulsed with 0·2 ml 2% colchicine (BDH) in saline, and incubated for a further 2 h at 37°. The cells were removed from the flask by trypsinization, and processed through hypotonic KCl and fixative (Hungerford, 1965). Chromosome spreads were made on clean chilled slides and stained for 10 min with a 1/20 dilution of Giemsa.

Screening for virus production

All cell cultures at Pass 4 or 5 were labelled with ^{3}H-thymidine and ^{3}H-uridine for 24–48 h followed by concentration of the culture supernatant and sucrose density gradient centrifugation as outlined by Norval and Marmion (1976). In one case, ST-4, the primary tumour disaggregated by the use of trypsin and the resulting single cells cultured in the presence of ^{3}H-thymidine and ^{3}H-uridine directly.

In addition, for the ST-6 cells only, the cultures at Pass 10 immediately before labelling were induced with IUDR (20 μg/ml) for 3 days followed by 2% DMSO for 3 days (Stewart *et al.*, 1972), mitomycin C (1 μg/ml) for 18 h in the dark (Weiss *et al.*, 1971), or cycloheximide (10 μg/ml) for 16 h (Aaronson and Dunn, 1974).

ST-1, ST-2 and ST-3 cells and cell lysates from Pass 2 were also examined by co-cultivation and infection of human embryo fibroblasts, RK_{13}, HEp-II and Vero cells followed by microscopic examination over a period of 4 weeks for evidence of any viral cytopathic effect.

RESULTS

Morbid anatomy and light microscopy of tumour cases

All 11 cases of suspected adenocarcinoma occurred in female sheep and they were from a variety of breeds including Blackface, Border Leicester and Halfbred. Their ages by dentition and skeletal

TABLE I.—*Source and investigation of cells cultured from explants of suspect adenocarcinoma of the small intestine of female sheep*

Case No.	Age of host	Tissue used for explant	Time between slaughter (S) or death (D) and culture		Morphology of explant culture
ST-1	Adult	Primary	I	(S)	A
ST-2	Adult	Mesenteric lymph node	I	(S)	A
ST-3	Adult	Primary	I	(S)	A
ST-4	Adult	Primary	II	(S)	Fungal contamination
ST-5	Adult	Primary	I	(S)	No growth
ST-6	ca. 9 months	Posterior mediastinal lymph node	I	(S)	B
ST-7	ca. 9 months	Flank lesion. Rumen serosa	II	(S)	C C
ST-8	Aged	Diaphragm lesion. Posterior mediastinal lymph node	II	(S)	B No growth
ST-9	Aged	Intestinal serosa	II	(S)	C
ST-10	Aged	Primary Flank lesion Ascitic fluid	II	(D)	No growth No growth No growth
ST-11	Adult	Flank lesion Intestinal serosa Rumen serosa	I	(S)	A A C

Key: A = Large fibroblast-like cells with stretched out processes; B = mixture of two sizes of epithelial-like cells, large fibroblast-like cells with long processes and long thin fibroblasts; C = long thin fibroblasts; I = on same day as animal's death; II = on the day following the animal's death.

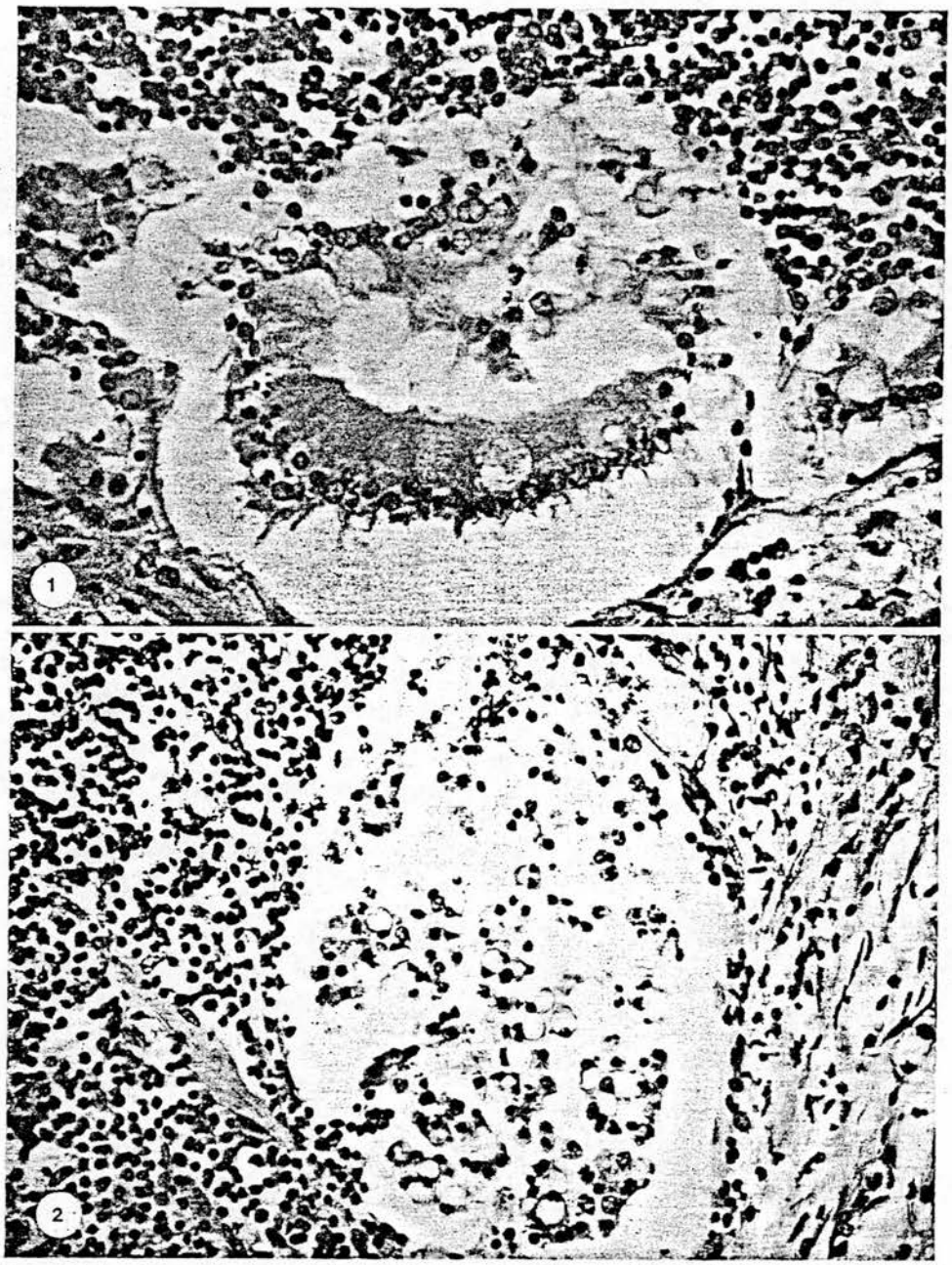

FIG. 1.—Photomicrograph of a metastasis in the subcapsular sinus of the posterior mediastinal lymph node of ST-6. Note the acinar pattern with basal nuclei and goblet-cell formation. Paraffin wax. H. & E. ×456.

FIG. 2.—Photomicrograph of metastatic cells in the subcapsular sinus of the posterior mediastinal lymph node of ST-6. Note many of the cells are signet-ring cells and there is some perinodal fibrosis. Paraffin wax. H. & E. ×380.

development ranged from 9 months through adult (over 4 years old) to aged (probably 7 years old or more) (Table I).

Ten of the cases were slaughtered at the abattoir, the eleventh died from respiratory distress caused by metastasis to the pleura and subsequent hydrothorax (ST-10). Morbid anatomy and histopathology revealed 10 of the cases were typical adenocarcinoma of the small intestine; in the eleventh (ST-7) the peritoneal lesions proved to be due to a nodular fibrous peritonitis probably associated with migrating helminth larvae.

The tumour cases had an annular stenosing primary lesion. The mucosal surface was usually ulcerated and the tumour in the mucosa formed ill-defined tubules, the cells having lost their nuclear polarity. The serosa at this site showed fibrosis with few tumour cells. Lymphatic metastases caused spread in the serosa proximal to the tumour and also to the drainage mesenteric lymph node. Transcoelomic metastases to the diaphragm and flanks were always present and from these sites lymphogenous involvement of the posterior mediastinal lymph node occurred. The peritoneal tumour sites were mainly composed of fibrous tissue rich in fibroblasts but showing few groups of tumour cells which sometimes became organized into an acinar structure with basal nuclei. Both these cell patterns could produce mucin either as signet-ring cells or sometimes as mucin-producing acini. The posterior mediastinal lymph nodes had less perinodal and medullary fibrosis than the mesenteric lymph nodes but both showed isolated tumour cells and a few well organized tumour-cell acini in the subcapsular sinus (Figs 1 and 2). Mitotic figures were rare in the epithelial tumour cells and were not found in the fibroblasts. The rumen was not always available for inspection but, in those cases where it was examined, half had rumen fibropapilloma present.

Primary culture of tumour material

At first, in addition to explant culture, the tissues were disaggregated by the use either of trypsin or of a mixture of collagenase and dispose, but it was found that explant cultures gave more satisfactory results with cells growing out from the tissue fragment within a few days. The initial cultures could be split into 2 after 2 weeks to 1 month. Thereafter, the rate of growth increased and the cells could be subcultured at a ratio of 1:3 every week. No cells grew from the ascitic fluid of ST-10 although initially some cells were present which looked like macrophages. Vigorous washing of the flank metastases was also tried in an attempt to dislodge surface tumour cells, but this was not successful. In one case, ST-4, the cultures became contaminated with fungi, and in another 2, ST-5 and ST-10, no cells grew out from the explants.

Morphology of cultured cells

The variety of cell types obtained on culture was of great interest. Typical fibroblasts grew from 2 specimens, ST-7 and ST-9, with long, thin, tapering morphology and which were contact-inhibited. In 4 cases, ST-1, ST-2, ST-3 and ST-11, the cell type which grew also looked fibroblastic although it was shorter and fatter than the fibroblasts already described. However, when the cultures were not confluent, the morphology was quite different. The cells were large and had stretched-out processes (Fig. 3a). As the cultures become more confluent, the cells seemed to be compressed into a fibroblastic shape (Fig. 3b).

The 2 remaining cultures, ST-6 and ST-8, were very different. In both there was a mixture of cell types which is illustrated in Fig. 4. It may be seen that there are patches of epithelial-like cells surrounded by fibroblast-like cells. This appearance was noticed in the primary culture from the explant (Fig. 5) and continued as a regular pattern throughout more than 30 subcultures which were performed weekly at a ratio of 1:2 or 1:3. In this respect, these cells may mimic the behaviour of cells within the tumour mass

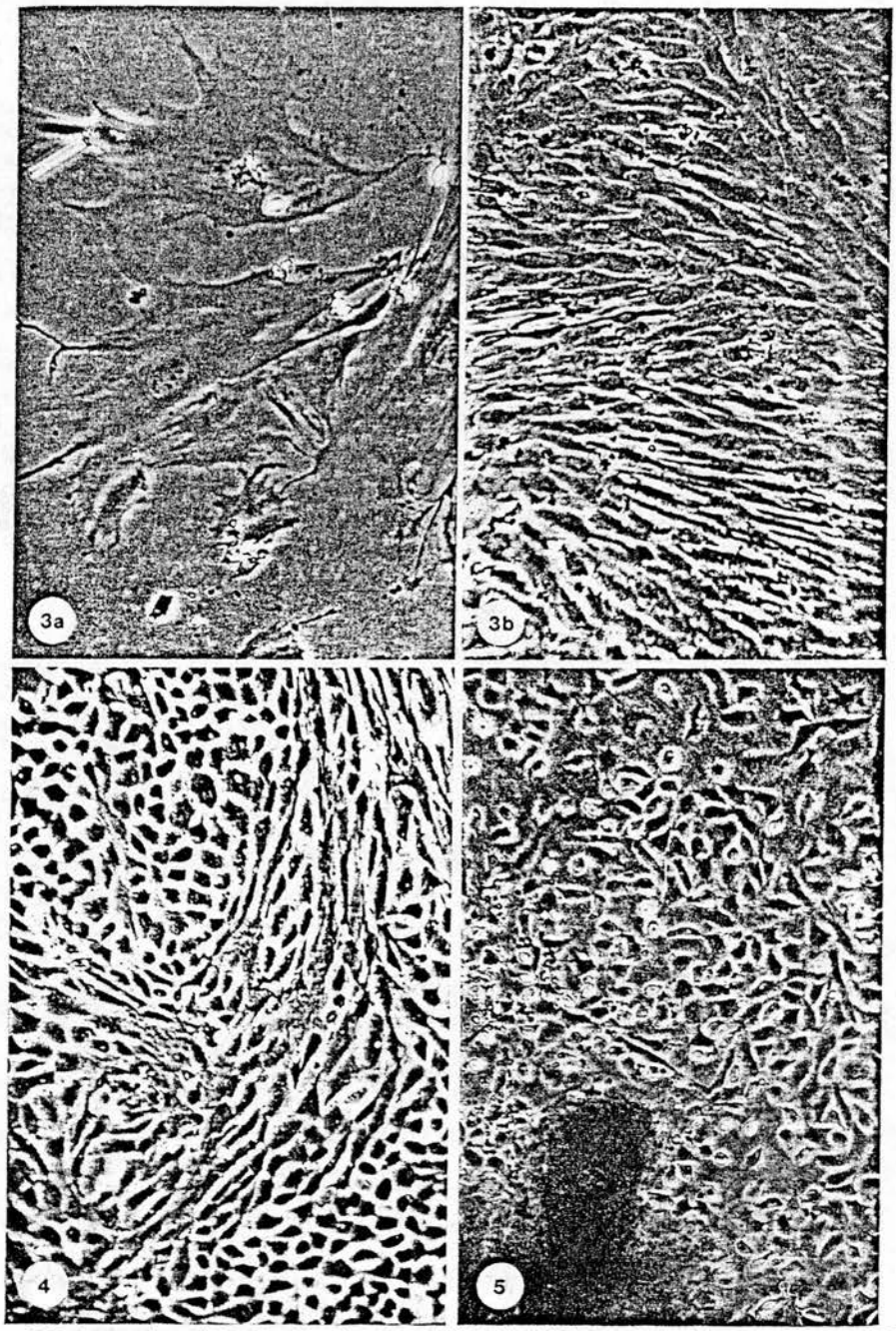

FIG. 3.—Cells from culture ST-1 (a) at low density and (b) when fully confluent. Phase contrast. × 75.
FIG. 4.—Cells from culture ST-6, Pass 3, showing mixed cell types. Phase contrast. × 75.
FIG. 5.—Explant culture of ST-6. Phase contrast. × 75.

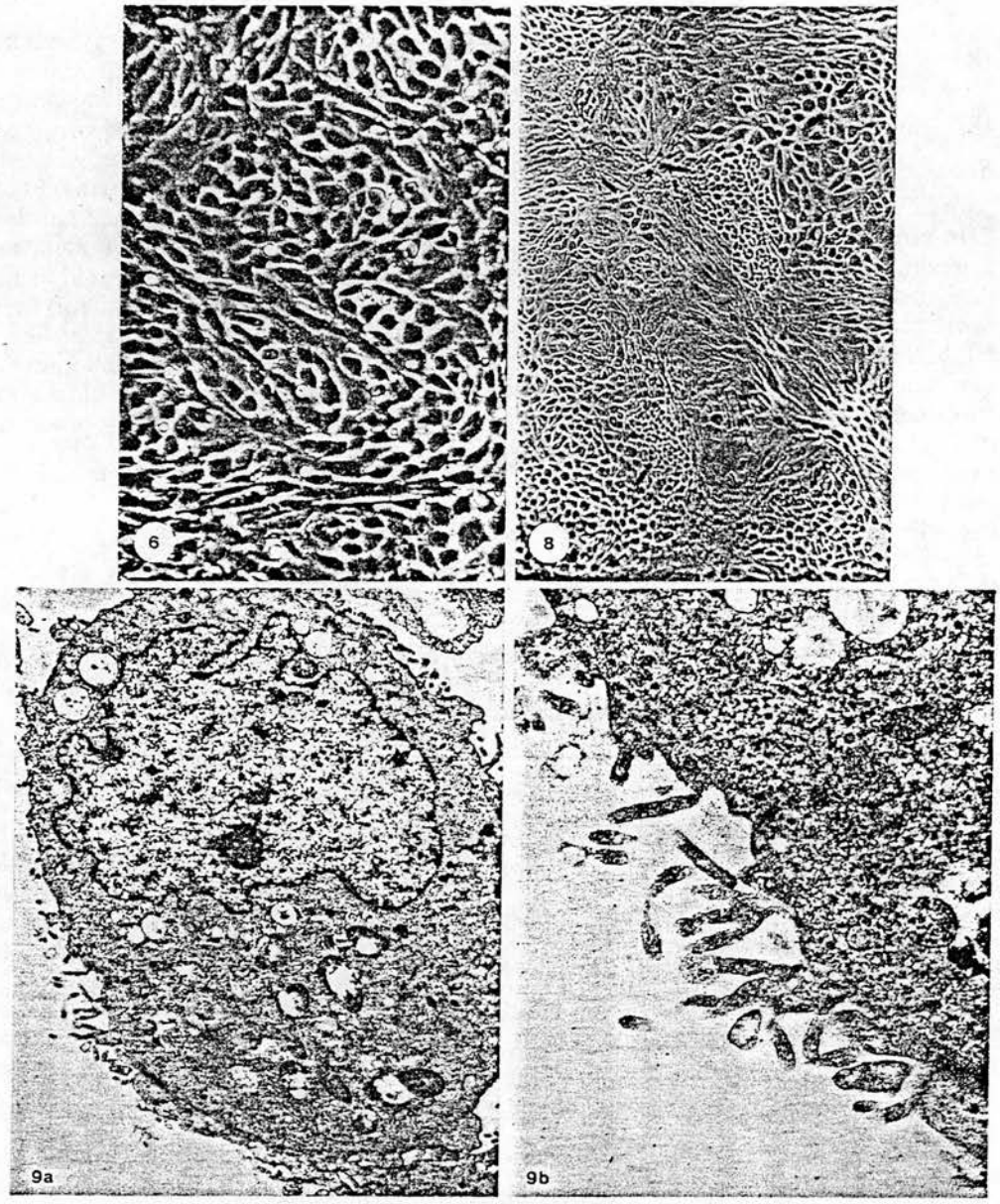

FIG. 6.—Fraction 4 cells from ST-6 after separation on BSA. Phase contrast. × 66.
FIG. 8.—ST-6 cells after 5 passages in Sun's modified medium with citrulline. Phase contrast. × 46.
FIG. 9.—Transmission electron micrograph of cell pellet from Fraction 4. (a) Typical cell; note numerous vacuoles with osmophilic material and prominent surface villi. × 4000. (b) Close-up of same cell showing surface villous structures. × 14,333.

and it was decided to attempt to separate the cell populations present within the ST-6 culture and to study their properties further.

Separation of ST-6 subpopulations

(a) *Culture in citrulline medium.*—The first approach was to culture the cells in a medium containing citrulline which was designed to inhibit the growth of fibroblasts as described by Sun *et al.* (1979). ST-6 cells were passaged 5 times in this medium and the appearance of the cultures compared with growth of these cells in Eagles' medium supplemented with the same serum.

In the modified medium with citrulline, the fibroblast-like cells gradually disappeared with each passage until none were seen after 5 passes. Two distinct populations of epithelial-like cells which differed in size remained, as shown in Fig. 8. The fibroblast-like cells persisted in the modified medium containing arginine and in the original Eagles' medium. It was very striking that, during the course of passage in the modified medium, the ST-6 cells seemed to rearrange themselves into cell types and to grow in discrete areas.

(b) *Sedimentation through B.S.A.*—In the second method ST-6 cells at Pass 10 were sedimented through B.S.A. and aliquots collected, with the result shown in Fig. 7. Each fraction was cultured after washing the cells. Fractions 1, 2 and 3 showed fibroblast-like cells of the type already described, *i.e.* they were large and spreading at low confluence with long processes. These cell fractions were grown up separately and then pooled for subsequent investigations and identified as Fraction 1–3. Fraction 4, on the other hand, contained a very large percentage of epithelial-like cells and these are illustrated in Fig. 6. Fractions 5–8 contained a mixture of epithelial cells and fibrobasts like the original culture and the remaining fractions had cells of typical fibroblast appearance, no epithelial cells being present.

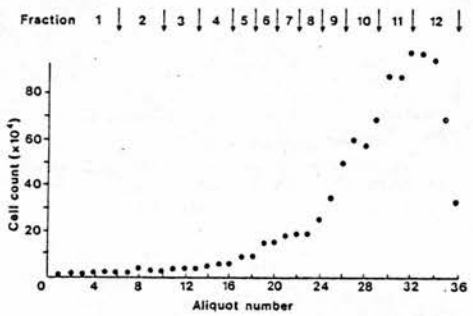

FIG. 7.—Profile of cell numbers in each aliquot after sedimentation of 10^7 ST-6 cells, Pass 10, through BSA solution. Fractions for culture were made from pooled aliquots as shown.

Ultrastructure of Fraction 4 and Fraction 1–3 subpopulations of ST–6

Thin sections of Fraction 4 cell pellets showed both individual cells and groups of cells. The nuclei were irregular in shape with strongly indented nuclear margins. where the cells had remained in groups, desmosomes were seen in tight junctional sites. At free cell surfaces, and where cells were loosely associated, there were prominent microvilli (Fig. 9). There were many cytoplasmic vacuoles which were apparently devoid of contents. At high power many of these structures contained sparse amounts of osmophilic material only, whilst others contained granular, more strongly osmophilic material. Relatively few mitochondria were present and these were usually degenerate. Other cells showed larger, more irregular vacuoles containing finely particulate, weakly osmophilic material characteristic of mucus. Some of the larger structures had villus-like cytoplasmic processes extending into their lumina. SEM observations showed numerous microvilli on the surface of Fraction 4 cells (Fig. 10a).

Examination of Fraction 1–3 cells by TEM showed cells with irregular, indented nuclei. Cell membranes were irregular but had markedly fewer microvilli than the Fraction 4 cells (Fig. 11). Round, and more irregular, vacuoles were observed in the endoplasmic reticulum. In addition, many cells showed accumulations of fibrillar material. The SEM appearance of a typical

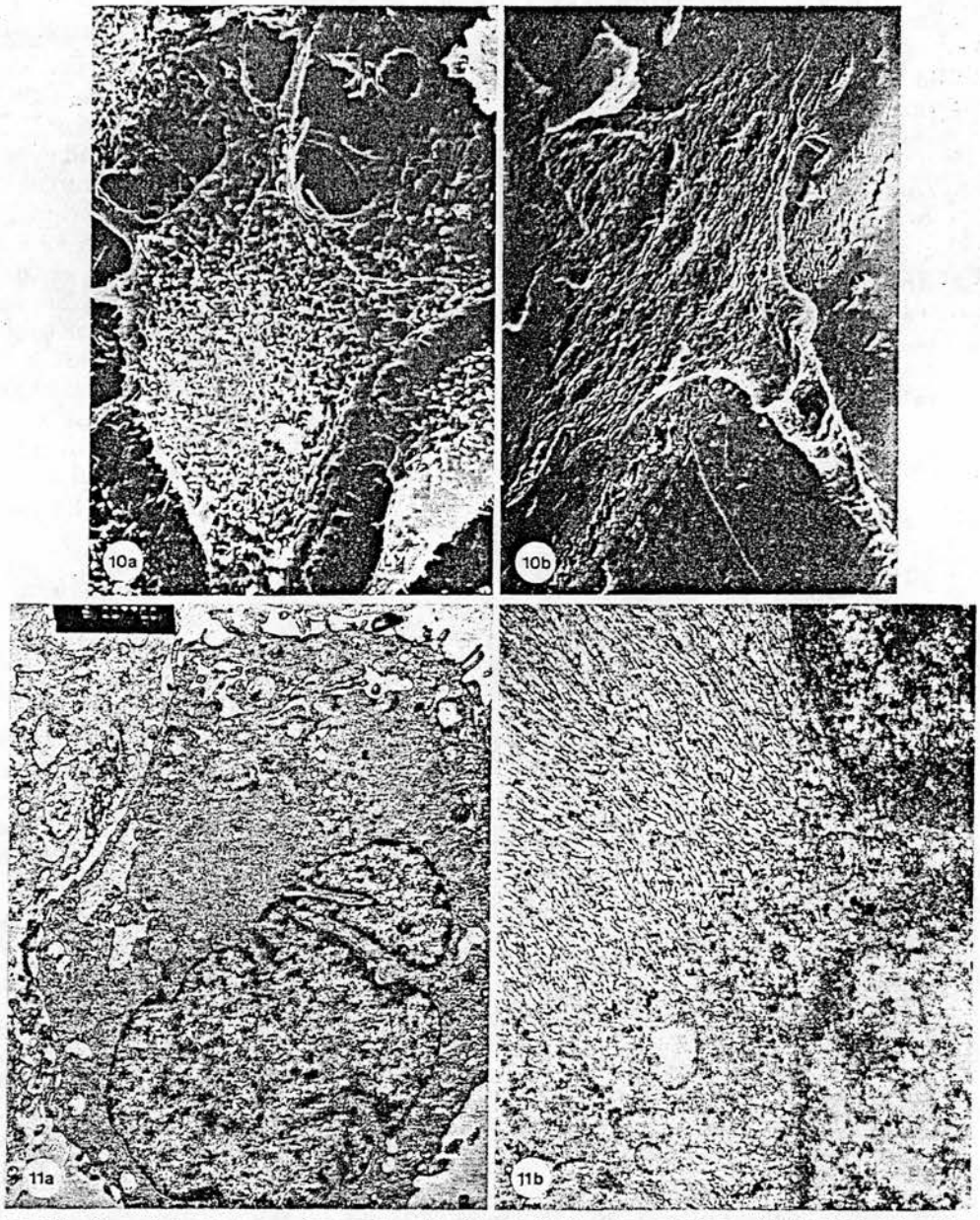

FIG. 10.—Scanning electron micrographs of cultured ST-6 subpopulations. (a) Typical cell with multiple villous processes on the cell surface from Fraction 4. Note apparent cell processes due to shrinkage artefact. × 2000. (b) Typical flattened cell with long cell processes and relatively smooth cell surface from Fraction 1–3. × 2000.

FIG. 11.—Transmission electron micrograph of cell pellet from Fraction 1–3. (a) Typical cell with filamentous inclusions. × 5132. (b) Close-up of same cell showing filamentous structures. × 30,666.

Fraction 1–3 cell is shown in Fig. 10b. No microvilli were seen on these cells and the cytoplasmic processes were extensive and, clearly, not due to shrinkage artefact.

No evidence of viral particles was observed in any of the sections examined.

Cultural characteristics of ST-6 and its subpopulations

(a) *Growth rate.*—The growth rate of Fraction 1–3, Fraction 4 and ST-6 cells was compared in the presence of 5% and 0·5% newborn calf serum, and the time taken for a cell division to occur was calculated. This is shown in Table II, in

TABLE II.—*Generation time of ST-6 cells and its subpopulations, Fraction 4 and Fraction 1–3 cells, in Eagles' medium containing 5% and 0·5% newborn calf serum*

	Generation time in 5% serum (days)	Generation time in 0·5% serum (days)
ST-6 (Pass 20)	2·8	3·6
Fraction 1–3 (Pass 5)	3·0	Did not divide
Fraction 4 (Pass 5)	1·2	1·6

which it may be seen that Fraction 4 cells grew about 3 times as quickly as Fraction 1–3 cells in medium containing 5% serum. In addition, they grew well in medium containing only 0·5% serum, while the Fraction 1–3 cells did not divide under these conditions.

(b) *Fibronectin immunofluorescence.*—In addition to the growth rate, the presence of fibronectin was tested by indirect immunofluorescence on ST-6 cells and its subpopulations. The unseparated ST-6 cells revealed a mixture of fibronectin-positive and fibronectin-negative cells. Fraction 4 cells had little or no fibronectin staining, while the Fraction 1–3 cells remained strongly positive.

(c) *Culture in semi-solid agar.*—As a further test of the possible tumour origin of Fraction 4 cells, they were cloned in semi-solid agar, being seeded at a density of 5×10^4 and 10^4 per plate. These numbers gave rise to 12 and 3 clones respectively, giving a cloning efficiency of 2–3 cells in 10^4 Fraction 4 cells. The clones which grew out contained epithelial-like cells.

(d) *Chromosome analysis.*—Chromosome analysis of ST-6 cells from Pass 12 was performed on 30 well spread cells and 25 of these possessed 54 chromosomes, the normal diploid number for sheep. Three cells had 51 chromosomes, one 52 and one 53 but the same chromosome was not missing consistently in these. Complete karyotypes were set out on 4 of the cells containing 54 chromosomes and all were shown to be normal (Fig. 12). No marker chromosomes or structural abnormalities were seen in any of the cells examined. 400 cells in mitosis were looked at and 32% of these possessed twice the normal diploid complement.

Screening for virus production

All cell strains which grew from the explant cultures (see Table I) were examined at Pass 4 or 5 for the presence of virus by light microscopy and electron microscopy, and none showed any evidence of this. In addition, ^{3}H-thymidine and ^{3}H-uridine were added to these cultures at Pass 4 or 5 in an attempt to label any virus which might be released into the fluid phase of the culture without an apparent cytopathic effect. No labelled material was found on subsequent sucrose gradient centrifugation of the concentrated culture supernatant with a density typical of virus particles. Also, in the case of the ST-6 cells, treatment with IUDR, cycloheximide and mitomycin C before labelling did not induce any virus. Finally, using 3 cultures, ST-1, ST-2 and ST-3, no cytopathic effect was detected after co-cultivation with a variety of cell lines or infection of these with cell lysates.

No culture contained Mycoplasmas.

DISCUSSION

Explant cultures were used in an attempt to grow tumour cells from adenocarcinoma of the small intestine of sheep and a variety of cell types emerged.

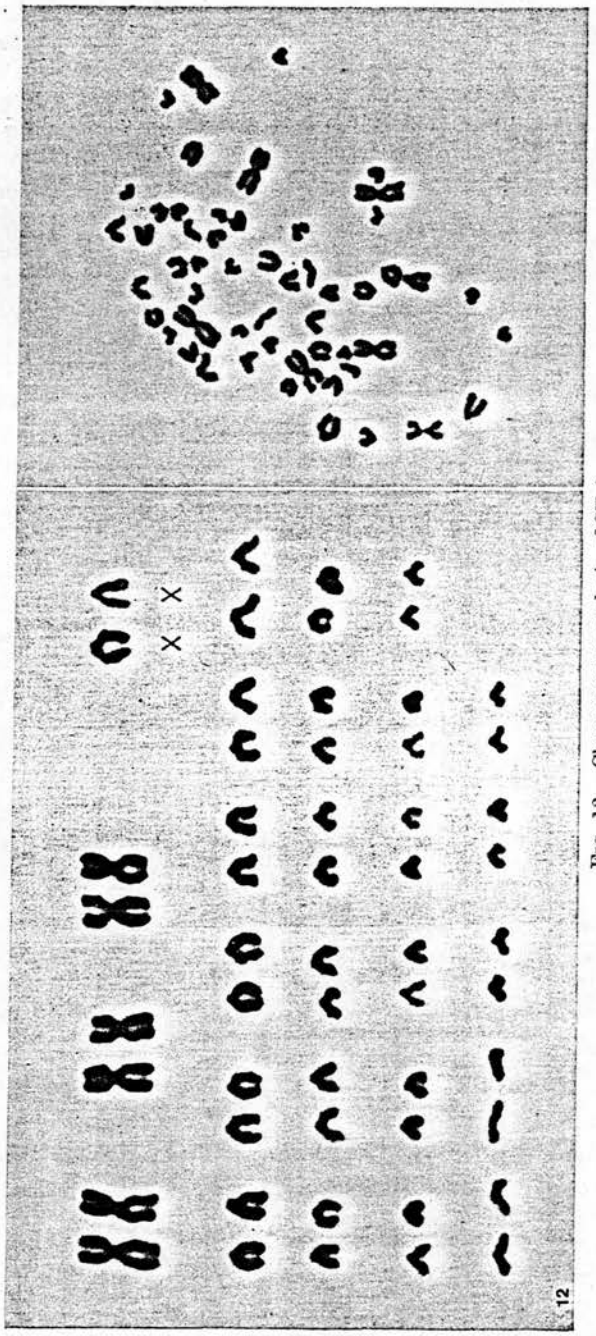

FIG. 12.—Chromosome analysis of ST-6.

Table I shows that the success of a culture could not be correlated with the speed of establishing the explant provided the sheep had been slaughtered and not left unopened for hours before postmortem examination. Likewise, the site from which the explant was taken did not seem to influence the successful outgrowth of cells. Three main cell types were found in the cultures: typical small fibroblasts, large fibroblast-like cells and epithelial-like cells. The small fibroblasts were recovered from both the non-neoplastic inflammatory reaction and from the tumour tissue. However, the fibrosis associated with the tumour when cultured soon after the death of the sheep often gave rise to large fibroblast-like cells. The fact that the most successful culture (ST-6) was obtained from a lamb might be taken to indicate that the case was unusual because of the age of the tumour-bearer. We do not think that this is tenable, first, because the other sheep to give a mixed epithelial-like and fibroblast-like culture (ST-8) was aged, *i.e.* at least 6 years old, and secondly, occurrence in lambs, although rare, has been previously reported; Georgsson and Vigfusson (1973), for example, stated that the youngest of their affected sheep was a yearling.

It has been suggested by Simpson and Jolly (1974) that this tumour arises from either adsorptive or goblet cells. Recently an ultrastructural study has been undertaken by Ross and Day (1979) and they reported that the tumour cells at the primary site, and in the metastases of the draining lymph node, form small acini which invade deeper tissue and provoke a fibrotic reaction. They suggested that, as all cell types found in the normal mucosa are present in the tumour, the lesion originates from undifferentiated intestinal epithelial cells.

This was of special interest in the present study as explants from 2 adenocarcinoma specimens gave rise to cell types of mixed morphology which persisted as such through several months of culture and over 30 passages. The cells seemed to form themselves into distinct growth patterns on subculture with the epithelial-like cells in discrete areas being surrounded by fibroblast-like cells. This mimics the appearance of the tumour *in vivo*. Separation of these cell types was achieved in 2 ways using methods which may also be applicable to other systems. The large fibroblast-like cells (Fraction 1–3) were observed ultrastructurally to have accumulations of fibrillar material in their cytoplasm together with mucin-containing vacuoles. It is not known what cell type they represent. On ultrastructural study, the epithelial-like cells (Fraction 4) showed extensive microvilli and frequent desmosomes between adjacent cell groups. In their cytoplasm there were vacuoles some of which contained mucus. On SEM cells of varying size could be seen as in the phase contrast examination of culture samples, but the morphology of these cells did not allow separation into 2 clearly defined subpopulations.

There were several factors which may indicate the oncogenic nature of these epithelial-like cells. In the first place, their growth rate was high and was little affected by reduction of the serum concentration to 0·5%. Secondly, a correlation has been drawn between the expression of fibronectin (LETS protein) and the oncogenicity of virus-transformed cell lines where transformed lines which produce tumours have little or no fibronectin, while in normal cells it is distributed over the surface (Chen *et al.*, 1976). An immunofluorescence test for the presence of fibronectin on the Fraction 4 cells showed little or no staining, while the Fraction 1–3 cells were strongly positive. In addition, the epithelial-like cells had the ability to form colonies in semi-solid agar. Therefore, it seems likely that these epithelial-like cells may represent the undifferentiated tumour cells described by Ross and Day (1979) and that they can be successfully cultured *in vitro*.

The original cells of this culture and all the other cell types which grew from explants of other specimens were screened for

virus production and all proved negative. It is possible that a virus is not involved in the aetiology of this tumour or, alternatively, that it may be present in a non-productive state, perhaps integrated into the host cell genome. The epithelial cells obtained here will be further studied to examine this possibility, especially with regard to ovine papilloma virus, in case the same correlation may apply in sheep as has been suggested in cattle (Jarrett *et al.*, 1978).

We gratefully acknowledge the technical assistance of Mrs Margaret Apps, and would like to thank Dr W. McBride and Dr M. Sharpe for helpful discussions. Grant support for this work was given by the Agricultural Research Council.

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Short Communication

THE CHARACTERISTICS OF A CANINE MAMMARY CARCINOMA CELL LINE, REM 134

R. W. ELSE*, M. NORVAL† AND W. A. NEILL†

*From the *Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH, and †Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG*

Received 16 March 1982 Accepted 16 June 1982

THE DOG has been widely used as an experimental animal for the assessment of human contraceptive and related drugs, with special reference to the relative risk of mammary carcinogenesis from such compounds (El Etreby & Gräf, 1979). In addition to the mammary dysplasias and neoplasias which may be induced experimentally, the domesticated canine has a significant incidence of naturally occurring spontaneous mammary neoplasia (Dorn *et al.*, 1968; Bostock, 1975). Between 40% (Else and Hannant, 1979*a*) and 60% (Misdorp & Hart, 1976) of bitches have malignant tumours leading to metastatic disease with euthanasia in the majority of cases—60% at 2 years after surgery in our experience.

Despite these facts, there are only 3 reports of tissue-culture studies of canine mammary carcinomas, by Cella (1967), Owen *et al.* (1977) and Watrach *et al.* (1978). Owen and co-workers reported the probable establishment of 2 cell lines: one, derived from a primary adenocarcinoma, was fibroblastic in nature, while the second, from a lung metastasis, was epithelial in nature.

This report describes the cultural and other characteristics of a cell line (REM 134) derived from a spontaneous primary canine carcinoma (Hampe & Misdorp, 1974). Representative tissue was fixed at the time of biopsy as 1–2mm cubes in 2·5% glutaraldehyde in cacodylate buffer and processed for examination in the transmission electron microscope as described previously (Else & Hannant, 1979*b*). Subsequently the bitch was killed 1 month after mastectomy and metastatic tumours involving the liver, diaphragm and lungs were seen at necropsy performed 6 h *post mortem*.

The tumour was disaggregated mechanically, and the fragments pressed through stainless steel mesh. After filtration through gauze to remove fatty fragments and debris, the cells were cultured using Parker TC 199 medium with the addition of penicillin (250 iu/ml), streptomycin (100 μg/ml) and 15% fetal calf serum. Cells were seeded at densities of $2{\cdot}5 \times 10^6$/ml and $5{\cdot}0 \times 10^6$/ml.

The cells were subcultured by trypsinization and, during an initial 10-month period, 112 passages were effected. Subsequently the medium was changed to Earle's-based Eagle's complete medium containing 100 iu/ml penicillin, 100 μg/ml streptomycin, 5% newborn calf serum and 2% fetal calf serum, and a further 25 passages carried out to date. Initially the doubling time, as estimated from total cell counts, was 48 h and at passage 120, 24 h.

Throughout the entire culture period the morphology of the cells was typically epithelial (Fig. 1). Their most striking feature at the light microscope level was the high number of cytoplasmic vacuoles.

Monolayer cultures were disaggregated with dispase (Boehringer) and mechanical scraping. After washing in 0·1M sodium

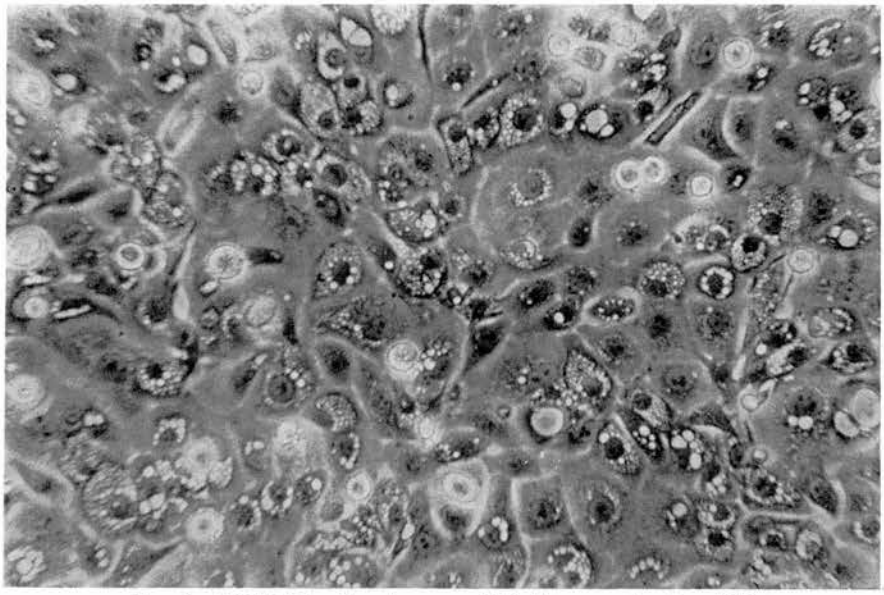

FIG. 1.—REM 134 cells at passage 115. Phase contract. ×100.

cacodylate buffer, cells were fixed in 2·5% glutaraldehyde for 2 h and pelleted by centrifugation at 3000 rev/min for 5 min. Cell pellets were post-fixed in 2% osmium tetroxide and embedded in Spurr resin. Ultrastructural examination of thin sections of cell pellets prepared from monolayers showed marked variation in cell size with varying nuclear–cytoplasmic ratios. Nuclei were frequently irregular and indented but were otherwise unremarkable (Fig. 2). Mitoses were often observed. Mitochondria were often either bizarre or apparently degenerate and relatively few in number. Osmiophilic round inclusion bodies were occasionally seen but only a few cells in any one sample showed large vacuoles with villous structures (Fig. 3). These latter structures are similar to intracytoplasmic duct-like vacuoles described in some human breast-tumour cell lines and primary tumours (Buehring & Hackett, 1974), and this is indicative of mammary epithelial origin. Many cells, particularly in the later passages, had abundant filaments of fine type or bolder tonofibrils (Fig. 4). A striking feature of the surfaces of cells was the presence of numerous well-developed microvilli (Fig. 2). These were also seen in the primary tumour where cells were loosely arranged. Although normal mammary epithelial cells have microvilli, the exaggerated formations here may be a reflection of the degree of

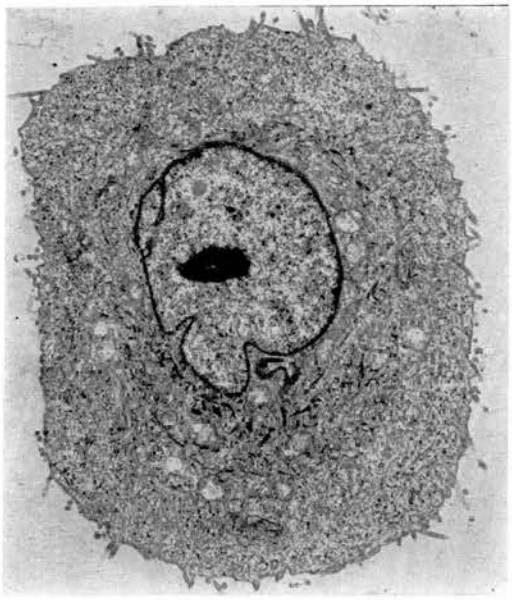

FIG. 2.—Typical REM 134 cell. ×4800.

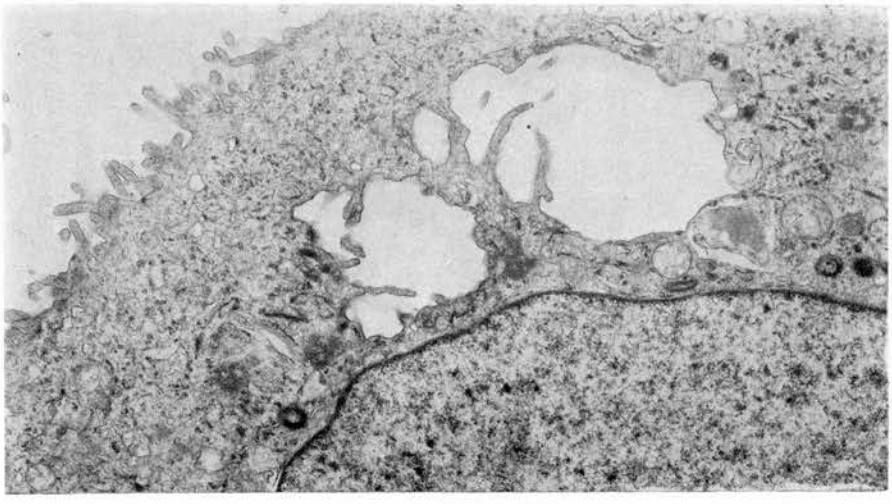

FIG. 3.—Vacuole-like structure with microvilli in a typical cell. ×45,000.

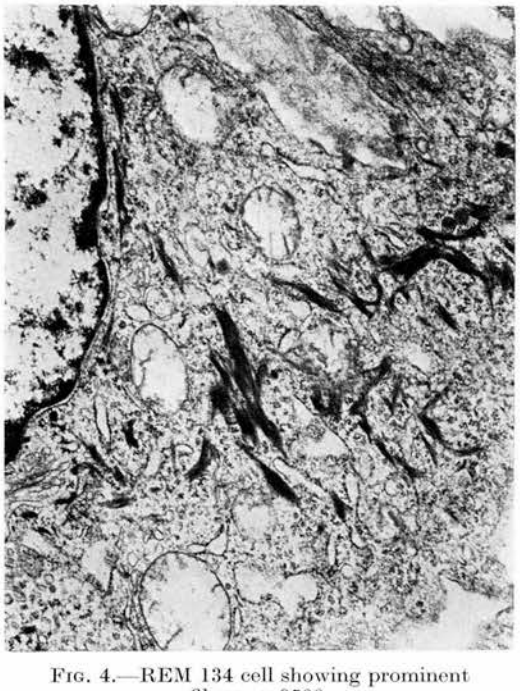

FIG. 4.—REM 134 cell showing prominent fibres. ×9500.

malignant transformation. Alternatively, they may be related to the mechanical nidation of metastatic cells. Cell pellets gave no information on desmosome status; monolayer preparations showed relatively few desmosomes. None of the cultural cells showed myosin bundles in their cytoplasm. There was no evidence of viral particles in any of the samples examined.

Monolayers were examined in a scanning electron microscope after fixation in 2·5% glutaraldehyde, followed by dehydration with acetone and critical-point drying. Monolayers had confluent cells with indistinct cell borders, raised centrally placed smooth nuclei, and prominent surface microvilli (Fig. 5) covering the remainder of the cell surface.

Cells for chromosome analysis were pulsed with 0·2 ml of 2% colchicine (BDH) in saline at 16 h after subculture from confluent monolayers and then incubated for a further 2 h at 37°C. The cells were removed from flasks by trypsinization and processed through hypotonic KCl and fixative (Hungerford, 1965). Chromosome spreads were made on clean chilled slides and stained for 10 min with 1/20 dilution Giemsa. Chromosomal examination at early passage level indicated a karyotype which was typically canine, 78 chromosomes per cell. However, some cells contained irregular chromosome numbers of 105–110. At passage 120, chromosomal analysis showed an average of 130/cell

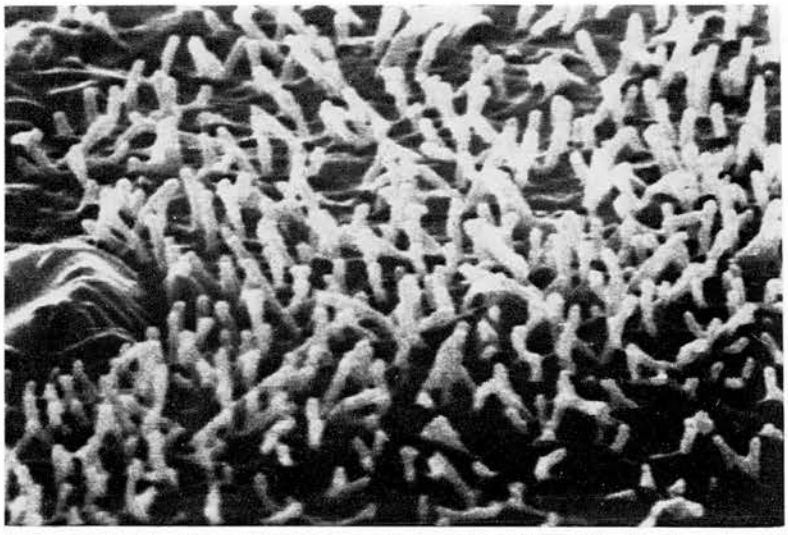

FIG. 5.—Scanning electron micrograph of REM 134 monolayer cell showing surface microvilli. ×10,000.

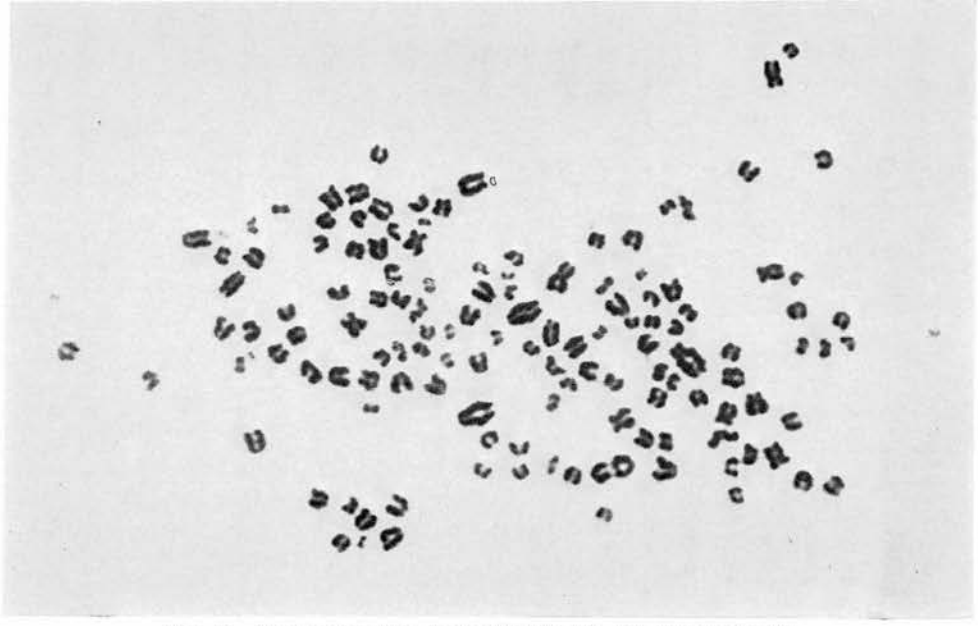

FIG. 6.—Chromosomal analysis of REM 134 cell at passage 120.

(range 124–136), one karyotype being depicted in Fig. 6.

The cells had the ability to grow in semi-solid agar (0·275% Seaplaque agarose, Marine Colloids Div.) and formed colonies easily visible by eye after 14 days' incubation. The cloning efficiency was ~4%. Individual colonies were picked using a micropipette and cultured separately; their properties are being determined at the present time.

There have been reports of retroviruses being associated with mouse mammary-tumour cell lines (Fine *et al.*, 1974),

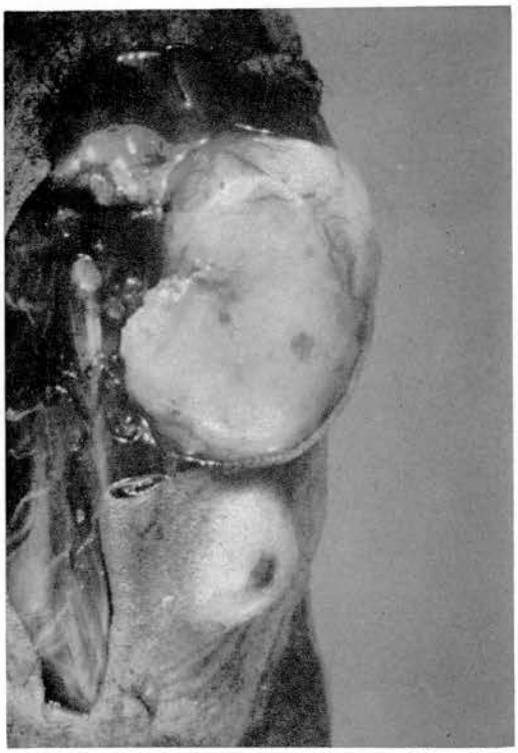

FIG. 7.—Typical tumour on dorsum of female "nude" mouse; 21 days' growth.

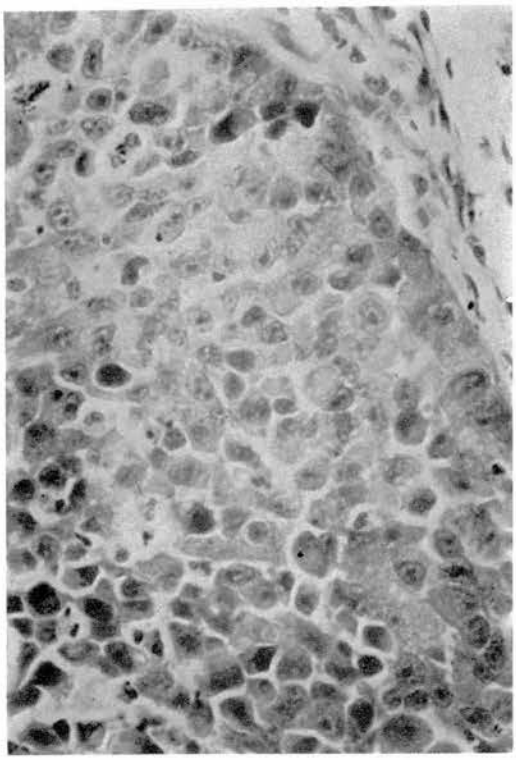

FIG. 8(a)

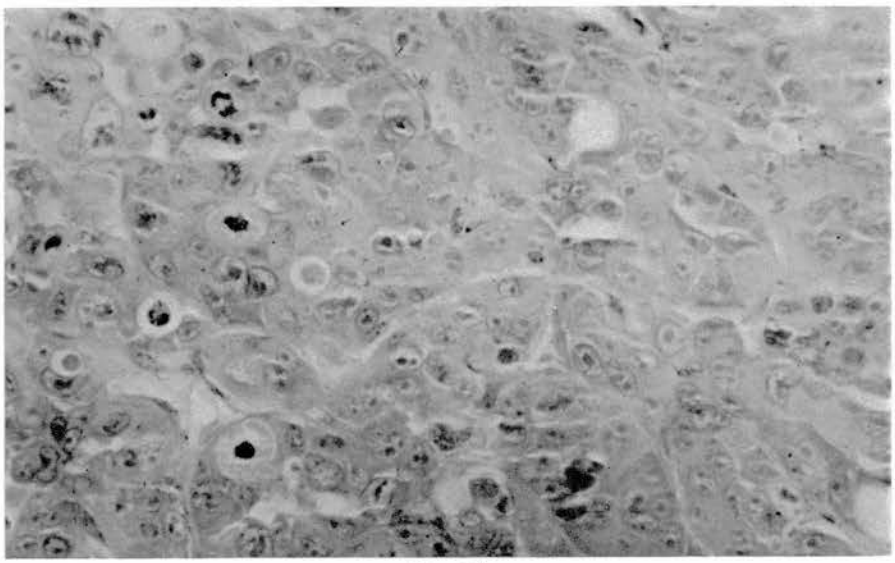

FIG. 8(b)

FIG. 8.—Histological appearance of tumours: (a) primary canine carcinoma, H. & E. ×1000; (b) "nude" mouse tumour. H. & E. ×2500.

human breast-cell lines (McGrath *et al.*, 1974) and a canine mammary-cell line (Watrach *et al.*, 1978). Thus to check for the presence of retroviruses in this canine mammary-cell line, labelling with [^{3}H]-uridine was carried out at passage 120, followed by concentration of the culture supernatant and sucrose density centrifugation as outlined by Norval & Marmion (1976). In addition, induction was attempted using progesterone (1 μg/ml, Sigma) or luteotropic hormone (5 and 10 μg/ml, Sigma) for 24 h before labelling with [^{3}H]-uridine. Levels of progesterone higher than 1 μg/ml were found to be toxic for the cells. No labelling was detected in areas of the sucrose gradients corresponding to a density of 1·16–1·18 g/ml, the reported density for most retroviruses. However, further studies are currently in progress on this question.

Cells from various passages were inoculated s.c. into 4-week-old female CBA Nu/Nu mice. Inoculation of 10^7 cells produced a solid tumour of diameter 0·4 cm, easily visible within 5 days, which continued to grow steadily until $\sim 2{\cdot}0$ cm in diameter, when the mice were killed at 21 days (Fig. 7). The short latent period of 5 days was a regular feature in all mice inoculated and contrasts with latency periods of $\geqslant 3$ weeks recorded for heterotransplanted human mammary carcinomas (Ozzello *et al.*, 1974). Presumably this feature is related to the highly malignant character of the cells. Inoculation of less than 10^5 cells produced no tumour.

Histologically the tumours were identical to the original primary canine carcinoma (Fig. 8). Some of the larger tumours induced by cells from later passages, however, also showed foci of tumour cells with bloated cytoplasm containing brightly eosinophilic fibrillary material. At cell junctions in these sites there was a lamellar arrangement of the material indicative of keratin-like formations. All samples examined histologically showed narrow compression capsules with sparse numbers of fibroblasts and polymorphonucleocytes migrating from adjacent blood vessels. There was usually evidence of local tissue infiltration by tumour cells at 21 days' growth. The largest tumours frequently had small central foci of necrosis. Such foci presumably reflect the rapid growth rate since the tumours generally had an abundant supply of thin-walled vascular channels. There is no evidence yet that any of the tumours metastasized but this aspect is under further study.

The tumours were excised, disaggregated with a mixture of collagenase and dispase (Boehringer) and 3×10^6 washed cells injected s.c. into female "nude" mice. Again tumours were visible within 1 week and grew rapidly. Serial tumour passage from one mouse to another was carried out 3 times and in each case the resulting tumours were histologically the same and similar to the original primary carcinoma. Culture of cells derived from disaggregation of these tumours yielded monolayers with the same morphology and karyotype as the original cells. In addition, they induced tumours when injected subsequently into female "nude" mice after 3 passages *in vitro*.

In male CBA "nude" mice inoculated with 2×10^6 cells, tumours did not appear before 19 days. The growth rate was estimated to be half that in the female mice with similar numbers of cells from the same passage. This observation may be indicative of hormonal control of tumour growth *in vivo*, and the apparent sex difference is currently under investigation.

Oestrogen-receptor assays of the primary tumour (Hamilton *et al.*, 1977) were positive. However, subsequent examination of the culture medium after growth of REM 134 cells at passage 125, by radioimmunoassay for oestrone and oestradiol-17$_\beta$, indicated no production of these steroids by the cells. In addition cultured cells and a solid tumour induced in a "nude" mouse showed no significant oestrogen or progesterone receptor activity as measured by uptake of [^{3}H]-oestradiol

(Hawkins, personal communication). It would appear, therefore, that *in vitro* the cell line is neither hormone-dependent nor capable of secreting detectable amounts of oestrogens. It is possible that for growth *in vivo* the cells may be dependent on some other factor such as pituitary hormones which may be released in higher amounts than the ovarian hormones.

In summary, this report describes a long-term culture derived from a canine mammary carcinoma which requires no special conditions for growth and appears to satisfy the criteria for an established cell line. One hundred and two canine mammary carcinomas have been cultured *in vitro* by one of us (R.W.E.), epithelial cells arising in about half this number. Out of all these, the REM 124 cells represent the only long-term line established which also has tumorigenic properties in "nude" mice. Furthermore, this cell line may provide a useful system for *in vitro* studies on mammary carcinoma cells as an alternative to dogs, particularly with reference to experimental hormonal manipulation.

We wish to acknowledge the assistance of Dr R. A. Hawkins, University of Edinburgh, in performing the hormone-receptor-assay studies.

This work was supported in part by the Cancer Research Campaign.

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Eur J Cancer Clin Oncol, Vol. 20, No. 12, pp. 1489–1500, 1984.
Printed in Great Britain.

0277–5379/84$3.00+0.00

Studies of Three Canine Mammary Carcinoma Cell Lines—I. *In Vitro* Properties*

MARY NORVAL,†‡ JEAN MAINGAY† and RODERICK W. ELSE§

†*Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K. and* §*Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH, U.K.*

Abstract—*Three cells lines, REM* 134, 111 *and* 367, *have been derived from canine mammary carcinomas and their morphological characteristics* in vitro *are described. They are tumorigenic in athymic nude mice, have no demonstrable fibronectin on their cell surfaces and exhibit a varied pattern of lectin binding. They can be cloned in semi-solid agar. One line, REM* 134, *responds to oestrogen and luteotropic hormone* in vitro, *although none of the three had demonstrable oestrogen receptors.*

INTRODUCTION

THERE is a high incidence of naturally occurring mammary carcinomas in bitches [1], and a recent survey of such tumours showed them to be remarkably similar to human tumours in their pathology and receptor status [2]. The bitch has been used as an experimental animal for the testing of human drugs, especially steroid hormones which may increase the risk of mammary carcinomas [3]. Depite this, there have been few reports of *in vitro* long-term culturing of cells from these carcinomas. Owen *et al.* [4] established two cell lines, one fibroblastic in nature, derived from a primary adenocarcinoma, and the other epithelial, from a lung metastatis, and described some of their properties in tissue culture. The induction of retrovirus particles from another canine mammary carcinoma line, after treatment with IUDR and dexamethasone, has also been reported [5]. Recently serial transplantation of four canine mammary tumours in athymic nude mice has been described [6], in addition to a study of the oestrogen receptor status of such experimentally induced tumours [7].

During this work, cells have been cultured from over 130 canine mammary carcinomas using various techniques of explantation and disaggregation. Some specimens yielded cells which grew *in vitro* with epithelioid morphology but only in three instances have cell lines been established which have properties of tansformed cells and which induce tumours in athymic nude mice. These were called REM 134, 367 and 111. The initial characterization of REM 134 cells has already been reported [8]. This communication compares the three lines with special regard to their morphology at the light and electron microscopic levels, their growth in semi-solid agar, their surface properties such as fibronectin content and lectin binding, assay for retroviruses and their stimulation by various hormones. Their tumorigenicity in athymic nude mice is reported in the accompanying paper [9].

MATERIALS AND METHODS

Clinical specimens

Mammary carcinomas and various metastases were routinely obtained from bitches at surgery or within 1 hr of euthanasia. All were classified subsequently according to the WHO International Histological Classification [10]. Within 1–2 hr explant cultures of small fragments, about 1 mm^3, were made in Petri dishes (Sterilin), or small pieces of tissue were disaggregated in collagenase/dispase (Boehringer) according to the manufacturer's instructions for 1–2 hr at 37°C. Single cells were washed and cultured in Falcon flasks (Flow). Eagle's minimal essential medium (EMEM) supplemented with 100 iu/ml penicillin, 200 μg/ml streptomycin, 50 iu/ml fungizone, 100 μg/ml gentamycin and 100% foetal calf serum was used routinely. Incubations were carried out

Accepted 22 June 1984.
*Financial support was from the Wellcome Trust.
‡To whom requests for reprint should be addressed.

at 37°C in an atmosphere of 5% CO_2 in air. Once the cells were established, gentamycin and fungizone were omitted from the medium and the serum changed to 5% foetal calf serum plus 5% newborn calf serum.

Electron microscopy

Cells were harvested by gentle mechanical scraping or by using dispase. After washing in 0.1 M sodium cacodylate buffer, they were fixed in 2.5% glutaraldehyde for 2 hr and pelleted by centrifugation at 3000 ***g*** for 5 min. Pellets were post-fixed in 2% osmium tetroxide and embedded in epoxy resin prior to sectioning. Thin sections, stained with lead citrate and uranyl acetate, were examined in a Philips 400 TEM. For scanning electron microscopy cells were cultured on 10-mm diameter glass cover-slips in Petri dishes. When confluent, monolayers were fixed in 2.5% glutaraldehyde and prepared for scanning electron microscopy by critical point drying. Specimens were viewed in an ISI 60 or Philips SEM 505.

Growth rate

Cells (4×10^5) were seeded into 50-mm Petri dishes in 5 ml EMEM containing either 5% foetal calf serum plus 5% newborn calf serum, or 0.5% of each. On each day for the next 4 days the number of viable cells present were counted after removal with trypsin–versene.

Growth in semi-solid agar

Cells (10^3) were cloned in 5 ml EMEM containing 10% foetal calf serum and 0.25% agarose (Seaplaque, Marine Colloids Division) in 50-mm Petri dishes with 1 ml medium containing 0.55% agarose as underlay. One mililitre of fresh medium with 0.25% agarose was added after 10 days incubation.

Fibronectin immunofluorescence

An indirect immunofluorescent test to detect fibronectin was carried out using cells grown in monolayers or cryostat sections of tumours which were fixed in 2% paraformaldehyde [11]. Rabbit antiserum was produced by injecting purified human cold insoluble globulin into rabbits, and was kindly donated by Dr J. Kinross. It was used at dilutions of 1/20, 1/100 and 1/250, while the FITC anti-rabbit conjugate (Wellcome) was used at 1/16.

Lectin immunofluorescence

A direct immunofluorescence assessment using FITC-labelled lectins (E-Y Laboratories, California) was carried out on cells grown in monolayers or cryostat sections of tumours fixed in cold acetone for 10 min.

Induction method for retroviruses

Cell cultures were induced with luteotropic hormone (5 μg/ml, Sigma) for 24 hr, progesterone (1 μg/ml, Sigma) for 24 hr and IUDR (20 μg/ml) for 24 hr, then dexamethasone (5 μg/ml) for 72 hr [5]. They were labelled with [^{3}H]-thymidine for 24–48 hr followed by concentration of the culture supernatant and sucrose density gradient centrifugation [12].

Effect of hormones on RNA synthesis

Cells (5×10^4) in 1.5 ml EMEM with 1% newborn calf serum and 1% foetal calf serum were put into tubes containing cover-slips. In some the medium was supplemented with luteotropic hormone in concentrations ranging from 10^{-6} to 10^{-12} M, or oestradiol-17β or testosterone from 10^{-5} to 10^{-11} M. Five microcuries of [^{3}H]-uridine was added to a duplicate set of tubes on the same day and to equivalent sets after the tubes had been incubated for 1, 2 and 3 days. The cover-slips were removed 24 hr after the addition of the label, washed in phosphate-buffered saline, fixed in cold 10% trichloroacetic acid for 10 min, washed in water, air-dried and counted in a PPO-toluene scintillator. An average of the two counts (which were always within 10% of each other) was taken.

Hormone receptors

Cultured cells of REM 134, 367 and 111 were examined for oestrogen and progesterone receptor activity as measured by uptake of [^{3}H]-oestradiol. Culture medium after growth of REM 134 cells was measured for oestrone and oestradiol-17β by a radioimmunoassay [Hawkins, personal communication]. In addition, the primary tumour of REM 134 has been assayed previously for oestrogen receptors [13].

Chromosome analysis

REM 134 and 111 were karyotyped using the method outlined in [8].

RESULTS

Origin of growth of REM 134, 367 *and* 111 *cell lines*

After explantation or disaggregation followed by culturing, epithelioid cells from canine mammary tumours were obtained in about 60/130 cases. In some of these the epithelial cells persisted only a short time, a month or two in culture, before detaching from the surface. Sometimes they became overgrown with fibroblastic cells or failed to grow on subculture. However, in some cases epithelial cells were obtained which could be subcultured successfully and maintained through at least 20 passages. Three, REM 134, 367 and 111, persisted as long-term cultures, considered as cell lines. Tumour

cells for REM 134 were obtained from a primary mammary solid carcinoma as described previously [8]. Cells for 367 were cultured from a primary papillary mammary adenocarcinoma obtained at the time of mastectomy. Tumour cells for 111 came from a pleural effusion collected immediately after euthanasia of a bitch with metastatic disease, following a primary mammary anaplastic solid carcinoma. All have been cultured for periods in excess of 1 yr; more than 130 passages of REM 134 cells have been effected and over 40 passages of 111. Cells of 367 exhibit a slower growth rate but have been passaged over 30 times. From the remaining epithelial cell cultures, two strains called A and B were used for comparison throughout, and one with fibroblastic appearance was also used.

The doubling time of REM 134 cells in 10% serum was about 24 hr, about 48 hr for 111 and 60 hr for 367 cells. If the serum content of the medium was reduced to 1%, the doubling time of REM 134 and 111 cells were unchanged for two divisions, and no growth occurred thereafter. The 367 cells grew very poorly in low serum.

The ability to form colonies in semi-solid agar has been reported as a good general index of transformation and subsequent tumorigenesis in suitable experimental animals [14]. Thus the three cell lines, as well as epithelial strains A and B and the fibroblastic strain, were cultured in semi-solid agar. Cells REM 134, 111 and 367 were found to form colonies visible by eye after 14 days of incubation which were picked using micropipettes and cultured separately. The cloning efficiency was 4% in all cases. No colonies were formed from the other cells strains.

Morphology of cell lines

The histopathological appearances of the original primary tumours REM 134, 111 and 367 are shown in Fig. 1, together with the cells from the pleural effusion, 111. Throughout the culture period the morphology of the three lines was typically epithelial (Fig. 2). REM 134 cells, however, showed considerable variation, ranging from polygonal to rounded. Epithelial cells from strain A and the fibroblastic cell strain used throughout are also shown in Fig. 2.

Marked variation in cell size was seen on thin sections of cultured cells, especially for REM 134. Nuclei were usually irregular, with indented nuclear membranes and coarse granular chromatin (Fig. 3a). Mitotic figures were common in REM 134 and 111, but less common in 367. Vacuole-like structures with villi were occasionally seen in REM 134 cells but were absent in 111 and 367. Many vacuoles in REM 134 cells contained electron-dense particles (Fig. 3b). Tonofibrils were common in the cytoplasm but mitochondria were relatively few and sometimes bizarre, particularly in REM 134. There were prominent microvilli in all three lines, but none showed myosin bundles. No evidence was seen of virus particles.

On examination of confluent monolayers in the scanning electron microscope, there were flattened cells with indistinct cell borders and slightly raised nuclear areas. At the periphery of confluent areas the cells were rounded and heaped (Fig. 4). The cells from the three lines showed prominent microvilli on their surfaces with fewer in nuclear zones. Rounded cells had prominent villous structures and some had bleb-like formations (Fig. 5). REM 134 cells had elongated villi (Fig. 6a), whilst both 367 and 111 had a greater density of shorter formations which were sometimes more bulbous (Fig. 6b).

The karyotype of REM 134 has already been reported [8]. Cells 111 at passage 10 showed marked polyploidy, with chromosome numbers ranging fron 126 to 167.

Surface properties

In addition to the ability to grow in suspension, most transformed cell lines exhibit a greatly reduced content or absence of fibronectin [11]. The cell lines and strains were tested by indirect immunofluorescence after growth on cover-slips. The results are shown in Table 1. It may be seen that REM 134, 367 and 111 cells had no detectable fibronectin, while the epithelial strains A and B and the fibroblastic strain had.

The surface of the cultured cells was also analysed by binding of nine different lectins using a direct immunofluorescent technique (Table 2). Wherever possible, the specificity of the binding was demonstrated by the reduction of immunofluorescence in the presence of the sugar to which the lectin attaches. There was no apparent pattern of lectin binding to separate the three lines from the other strains used.

Table 1. *Presence of fibronectin as measured by indirect immunofluorescence*

	Dilutions of antiserum		
	1/20	1/100	1/250
REM 134	–	–	–
367	–	–	–
111	–	–	–
Epithelial strain A	++	++	–
Epithelial strain B	++	+	–
Fibroblastic strain	++	++	–
Human embryo fibroblasts	+++	++	+

+++, ++ and + = degrees of immunofluorescence; – = no immunofluorescence.

Table 2. Binding of FITC-lectins to cell cultures as measured by direct immunofluorescence

	REM 134	111	367	Epithelial strain A	Epthelial strain B	Fibroblastic strain
PNA	–	–	–	–	–	–
GS-1	++	+	+	–	–	++
GS-I + D-gal	–	–	–	–	–	+
BSA-II	+++	+++	+++	++	++	+++
DBA	–	+	+	–	+	+
DBA + NAc-D-gal	–	–	–	–	–	–
SBA	+++	+	++	+	–	–
SBA + D-gal	–	–	–	–	–	–
MPA	–	+	–	–	++	+
MPA + D-gal	–	–	–	–	–	–
RCA	++	+	+	+	+	++
RCA + D-gal	–	–	–	–	+	–
WGA	+++	+++	+++	+++	+++	+++
UEA	–	–	–	+	–	–
UAE + L-fuc	–	–	–	–	–	–

PNA, *Arachis hypogaea* agglutinin; BSA-II, *Bandeiraea simpliciforlia* agglutinin; SBA, *Glycine max* agglutinin; RCA, *Ricinus communis* agglutinin; UEA, *Ulex europaeus* agglutinin; GS-I, *Bandeiraea simplicifolia* agglutinin; DBA, *Dolichos biflorus* agglutinin; MPA, *Maclura pomifera* agglutinin; WGA, *Triticum vulgaris* agglutinin; D-gal, D-galactose; L-fuc, L-fucose; NAc-D-gal, *N*-acetyl-D-galactosamine.
+++, ++ and + = degrees of immunofluorescence; – = no immunofluorescence.

Induction of retroviruses

There have been several reports of retroviruses being associated with mammary carcinoma cell lines from various species, such as human [15], murine [16] and canine, the latter being induced with IUDR followed by dexamethasone [5]. When such a regime was carried out on REM 134, 111 and 367 cells no virus particles were detected using [^{3}H]-uridine labelling. In addition, induction by treatment with progesterone or luteotropic hormone was not successful.

Effect of hormones on rate of growth and hormone receptors

Oestradiol-17β, testosterone and luteotropic hormone at varying molarities were added to REM 134, 367 and 111 to find out if they stimulated the growth rate. This was measured by the uptake of [^{3}H]-uridine over a 4-day period after the addition of the hormone. Cell lines 111 and 367 did not respond to any of the added hormones at any concentration. In the case of REM 134 there was no effect due to the addition of testosterone, but oestradiol and luteotropic hormone stimulated RNA synthesis 72 hr after addition by as much as 2–3 times, as illustrated in Fig. 7.

REM 134 and 111 cell lines were analysed for oestrogen and progesterone receptor activity and both were negative. In addition, the uptake of [^{3}H]-oestradiol (5 nM) into 5×10^8 REM 134 cells was measured in a 1- to 2-hr period at 37°C. The cells were washed once, incubated in oestrogen-free buffer for 0.5 hr and then washed three times prior to counting. Control cells were incubated with [^{3}H]-oestradiol plus a 500-fold excess of diethylstilbestrol, which competes for oetrogen binding sites and would therefore block receptor-binding. Three per cent of the initial radioactivity was found in the cells after incubation but the control cells contained the same amount, thus implying that there were no oestrogen receptors.

DISCUSSION

Three epithelial cells lines derived from canine mammary carcinomas have been described which show tumorigenic properties in athymic nude mice [9]. They have varied morphology but all contain detectable fibronectin on their cell surfaces and have the ability to grow in semi-solid agar, producing colonies at the same efficiency. The clones obtained seem to have identical morphology at the light microscopic level as the parent cells and are presently being tested for their

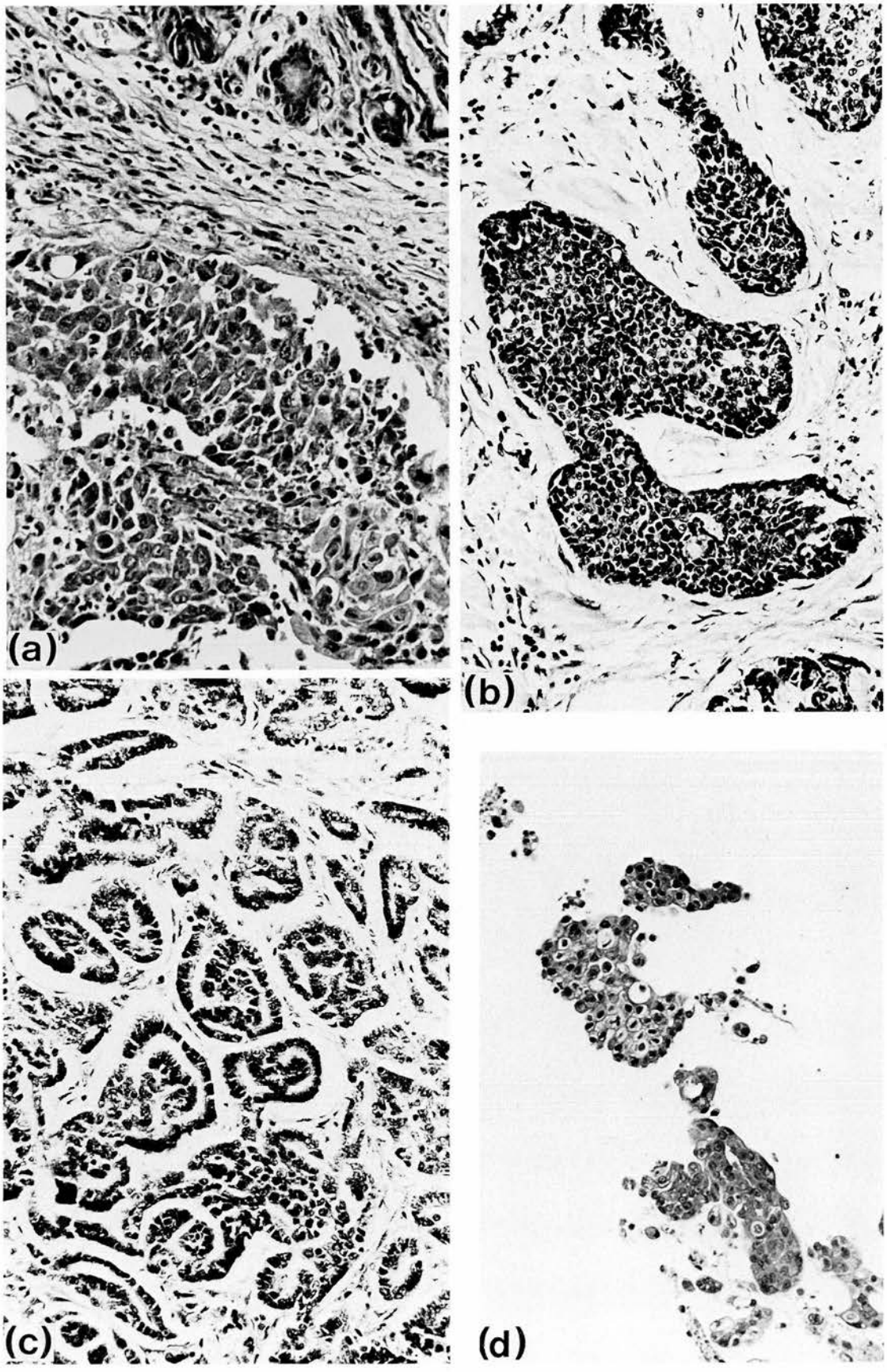

Fig. 1. *Histopathology of original primary tumours,* ×200, *H and E stain.* (*a*) *REM* 134; (*b*) 111; (*c*) 367; *and* (*d*) *pleural effusion* 111.

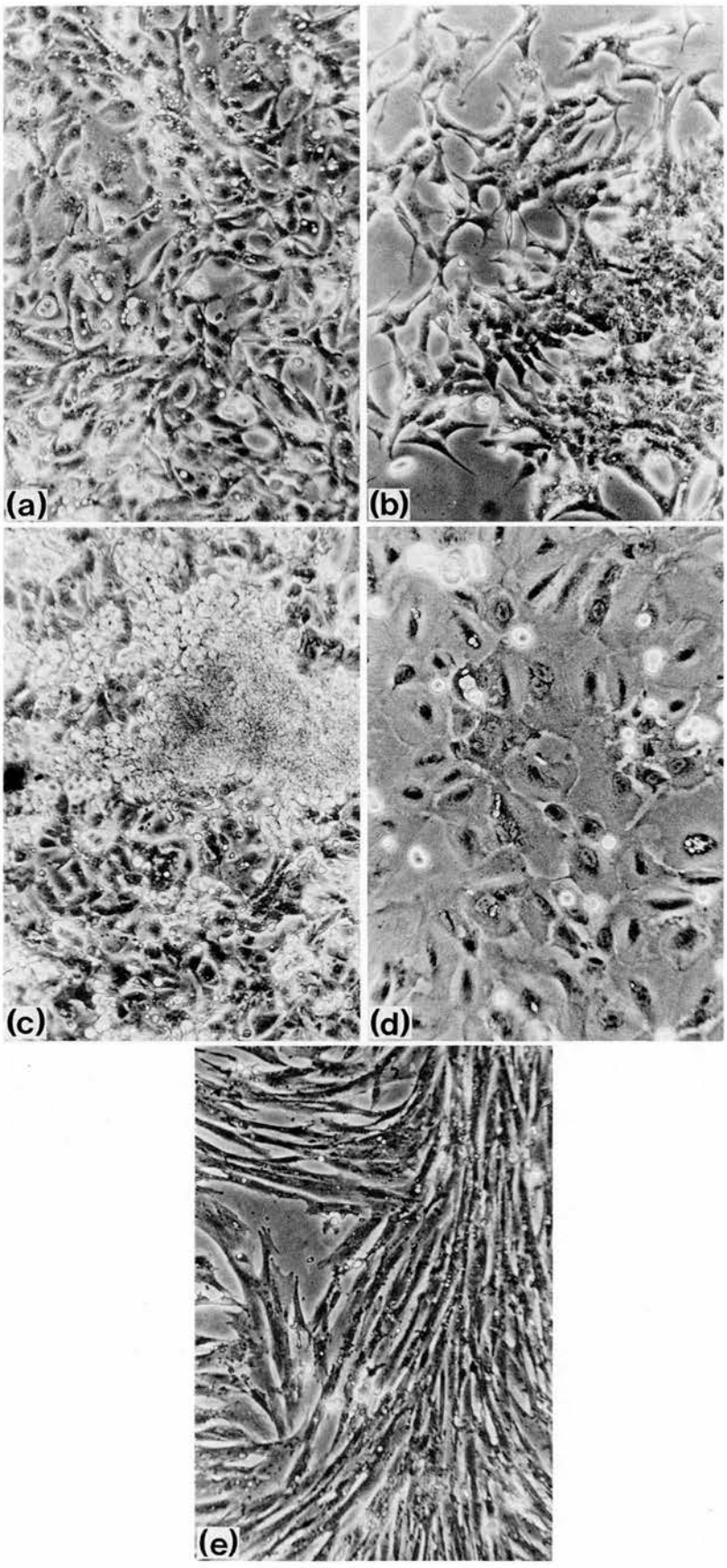

Fig. 2. Phase-contrast microscopy, ×100. *(a) REM* 134; *(b)* 111; *(c)* 367; *(d) epithelial strain A; (e) fibroblastic strain.*

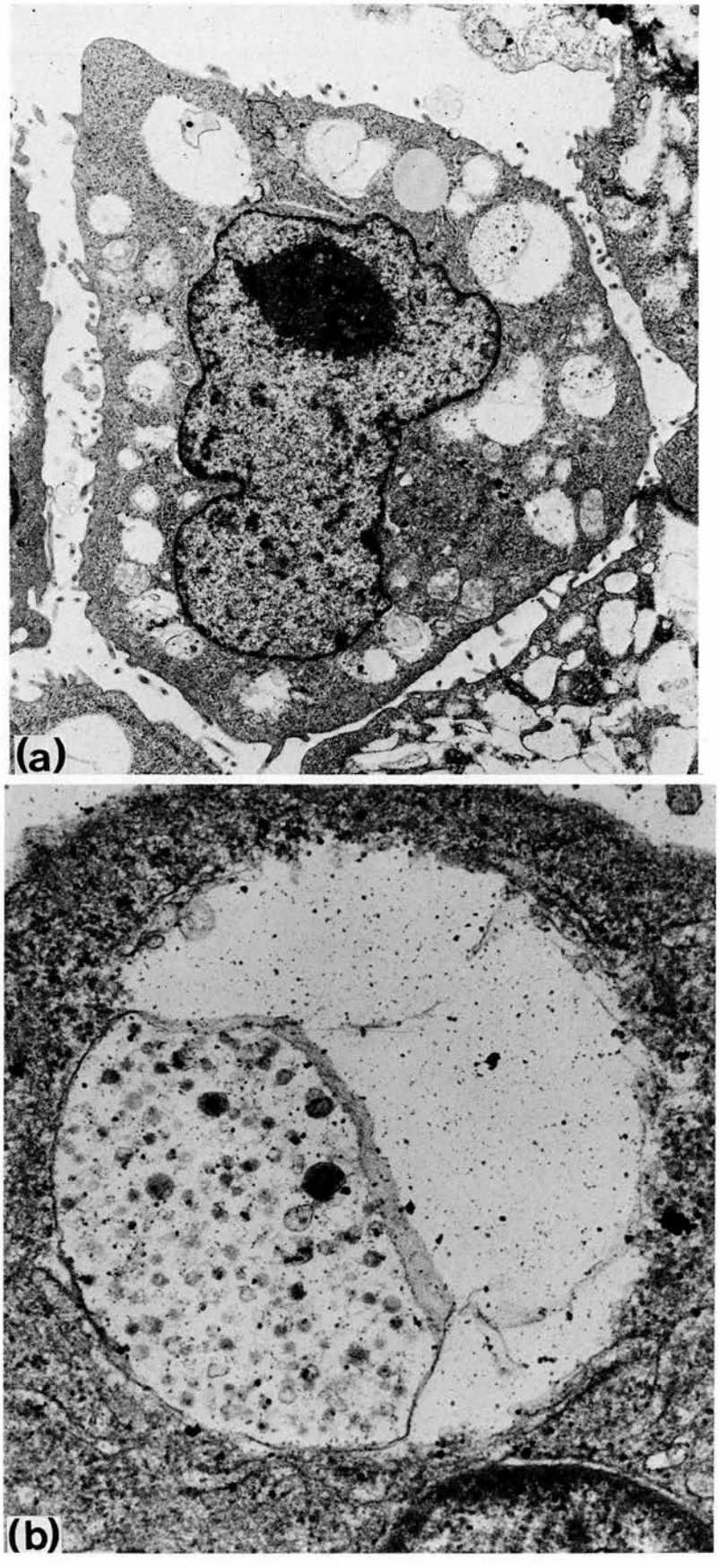

Fig. 3. Transmission electron micrographs of REM 134 cells. (a) Typical cell, ×10,000; *(b) vacuole-like structure containing particles,* ×71,000.

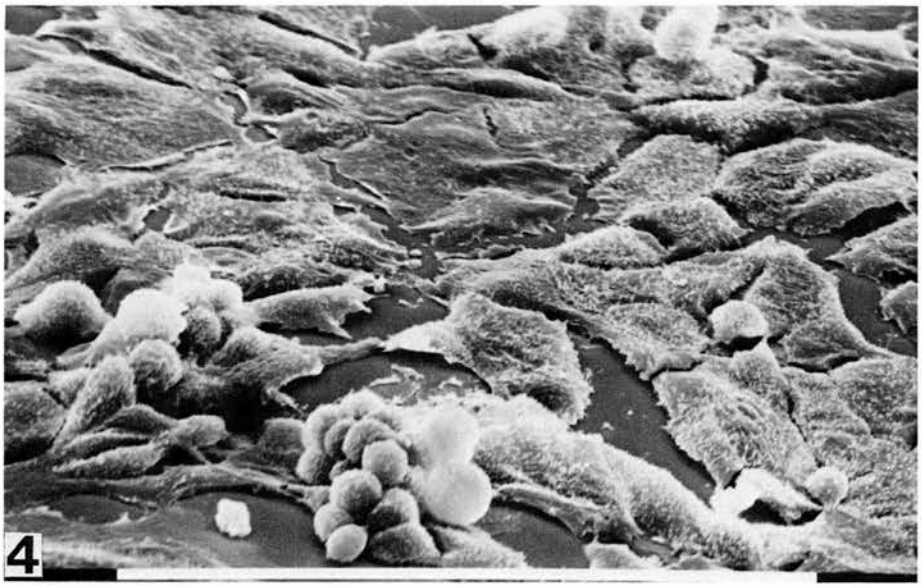

Fig. 4. Scanning electron micrograph (SEM) of 367 cell monolayer, showing rounding and heaping of cells at periphery, ×1850.

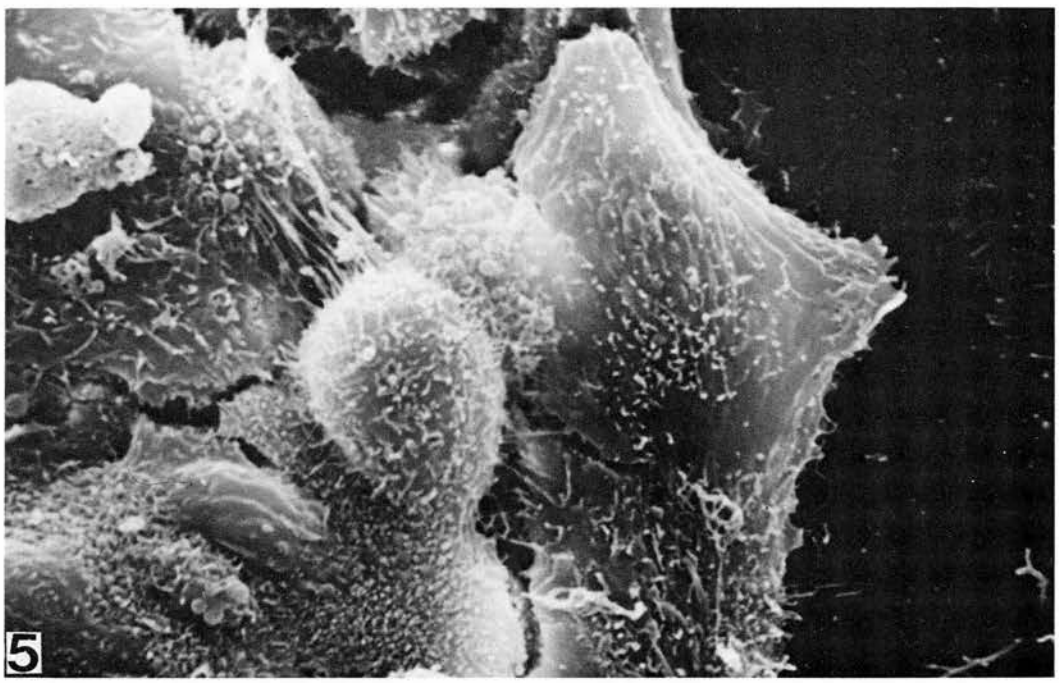

Fig. 5. SEM of 111 *cells showing villi and blebs on rounded cells,* ×4000.

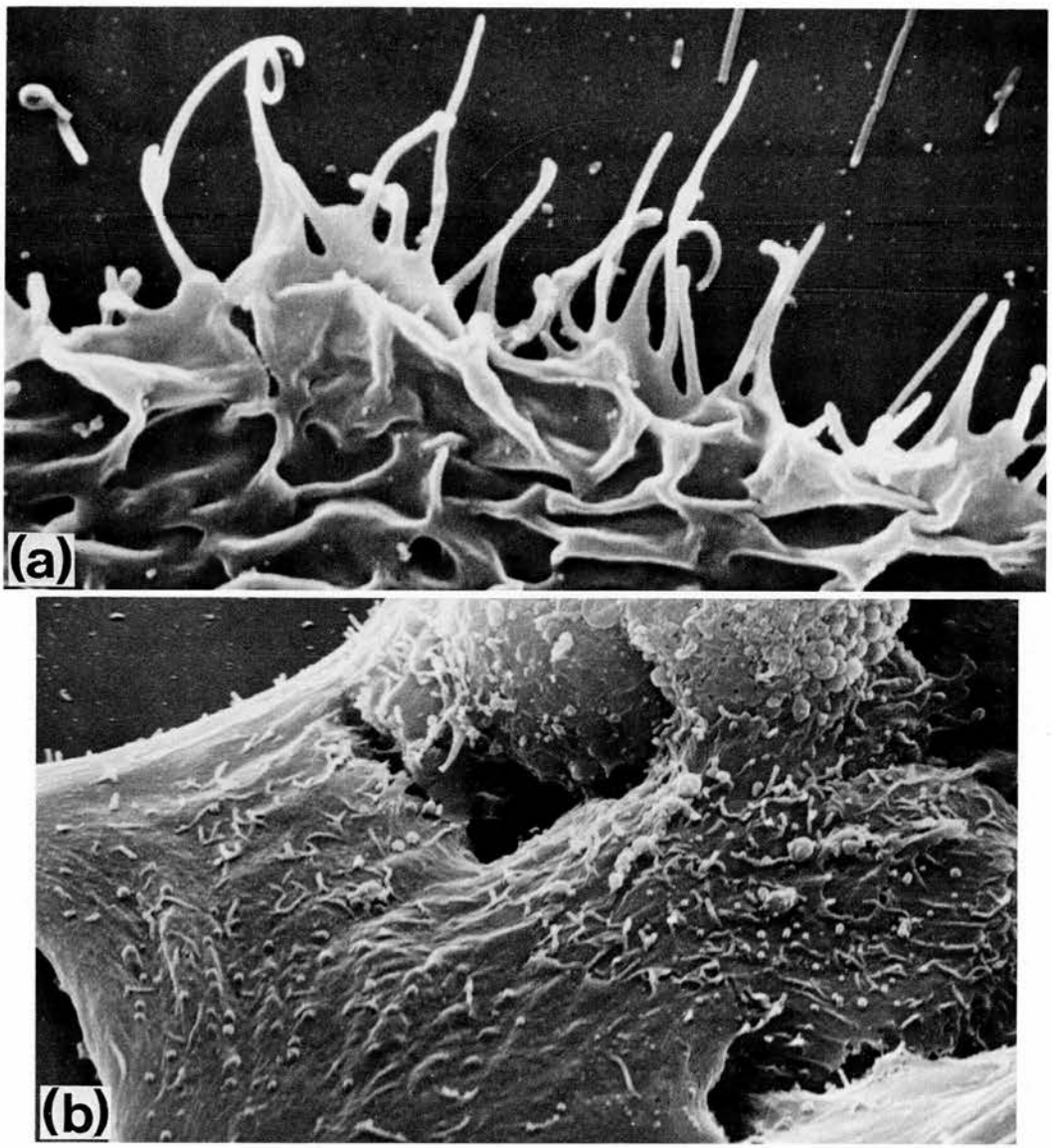

Fig. 6. SEM of villous structures on cells with elongated villi in (a) REM 134, and bulbous structures in (b) 367; ×11,200 and ×7000 respectively.

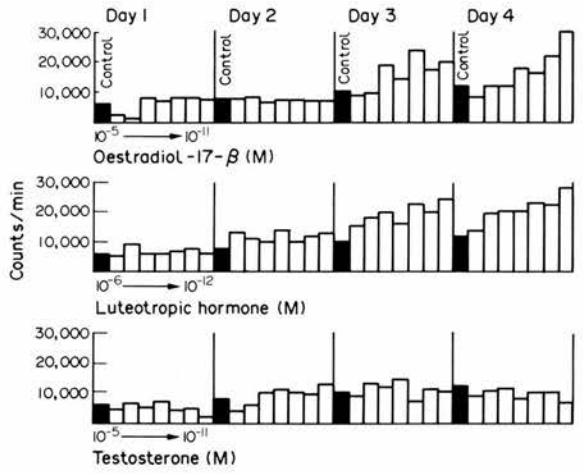

Fig. 7. Incorporation of [^{3}H]*-uridine in REM 134 cells over a 24-hr period on each of four days after the addition of oestradiol-17β, luteotropic hormone and testosterone. Concentrations of hormones are in ten-fold dilutions starting at* 10^{-5} *or* 10^{-6} M, *as shown. Controls for each day with no added hormone are also shown.*

tumorigenic properties and ultrastructure. While the REM 134 cells grew as thick even layers of cells after reaching confluence, 111 and 367 began to heap up at the edges of 'islands' of cells and these had a tendency to detach from the surface, although still viable and capable of starting re-growth in a new flask. The pattern of binding of lectins to the surfaces of the cell lines was not consistent and they could not be distinguished from the epithelial strains on this basis.

Attempts to demonstrate productive infection with retroviruses failed despite the same methods of induction being used as had been reported for a canine mammary carcinoma line [5] and for MCF-7 cells [15]. The lines are presently being tested with anti-gp52 of a murine mammary tumour virus [17]. In addition, an antiserum prepared in rabbits to the membranes of REM 134 cells is being used in Western blotting to detect similarities and differences in polypeptides eluted from the surface of REM 134, 367 and 111, epithelial cell strains and mammary carcinomas themselves.

Oestrogen receptors were detected on the original tumour from which the REM 134 cell line was derived [13]. The tumours which gave rise to 111 and 367 lines were not tested in this way. A recent survey has indicated the presence of such receptors on 61% of canine mammary carcinomas [2]. *In vitro*, no receptors were detected on the cell lines tested. However, while 367 and 111 were not stimulated by oestrogen, luteotropic hormone or testosterone in culture, REM 134 cells showed an increase in [^{3}H]-uridine uptake in the presence of oestrogen and luteotropic hormone at physiological levels. Thus there seemed to be a response of REM 134 cells to female hormones, although no receptor activity could be detected. This may be due to low levels of such receptors and an insufficiently sensitive assay system, or to some other reason. The rate of growth of tumours induced by the three lines in male and female nude mice is reported in the accompanying paper [9]

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Eur J Cancer Clin Oncol, Vol. 20, No. 12, pp. 1501–1508, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

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Studies of Three Canine Mammary Cell Lines—II. *In Vivo* Properties*

MARY NORVAL,†‡ JEAN MAINGAY† and RODERICK W. ELSE§

†*Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K. and* §*Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH, U.K.*

Abstract—*Three cell lines, REM* 134, 111 *and* 367, *derived from canine mammary carcinomas have been used to induce tumours in athymic nude mice after subcutaneous injection. The histopathology of the tumours was compared and each was found to resemble closely the original tumour. This did not change after serial* in vivo *passage. Metastasis never occurred. Injection of REM* 134 *cells intracranially resulted in a fast-growing tumour which also did not metastasize; injection intrapleurally resulted in growths most commonly on the mediastinum with confinement to the chest cavity. Fibronectin was present in the subcutaneous tumours. Two of the cell lines were cloned in semi-solid agar. When tested, these clones induced tumours identical histologically to the uncloned ones. Finally, male and female mice were injected subcutaneously with the same number of cells from each of the three lines but the rate of tumour growth did not differ significantly between the two sexes.*

INTRODUCTION

THE ORIGIN of three epithelial cell lines, REM 134, 111 and 367, derived from canine mammary carcinomas and their properties *in vitro* have been described in a previous paper [1]. This communication deals with their tumorigenicity in athymic nude mice. REM 134 cells have already been reported to induce tumours in nude mice and these could be serially transplanted [2]. There was the suggestion of a difference in the rate of tumour growth between male and female mice and this has been further investigated. Recently a study was made of four canine mammary tumours in which fragments of tissue implanted in nude mice induced formation of tumours which could then be serially transplanted [3]. The oestrogen receptors of the induced tumours were assayed [4].

MATERIALS AND METHODS

Cell lines

The origin, growth and maintenance of REM 134, 367 and 111 cell lines have already been described [1]. For inoculation into mice the cells were removed from the surface of the culture vessel with trypsin–versene, washed twice in medium, counted and re-suspended at the required concentration in 0.1 ml saline.

Mice

CBA athymic mice, 4–6 weeks old, were purchased from the Clinical Research Centre, Harrow, U.K. They were kept in an isolator with autoclaved bedding, filtered air, sterile food and acidified water (pH 2.8). Injections of cells were carried out subcutaneously, intracranially and intrapleurally. Tumour diameters in two directions (and height in some instances) were measured using Vernier callipers, and the mice were weighed at weekly intervals. When the tumours reached around 2 cm in diameter or became necrotic the mice were killed by cervical dislocation. The tumours were excised using an aseptic technique and, in some instances, disaggregated with a mixture of collagenase/dispase (Boehringer) before re-injection into another mouse as cell suspensions or before culturing *in vitro*. Part of each was fixed for histopathology in 10% buffered formalin, sectioned after embedding in paraffin wax and stained with haematoxylin and eosin. Various organs were also prepared in the same way. Pieces of tumour were prepared for electron microscopy

Accepted 22 June 1984.
*Financial support was from the Wellcome Trust.
‡To whom requests for reprint should be addressed.

by fixing in 2.5% glutaraldehyde and embedding in 'Transmit' resin (Taab Ltd).

RESULTS

Initially various numbers of cells from REM 134, 367 and 111 were inoculated subcutaneously into female athymic nude mice. REM 134 cells were found to be highly tumorigenic, an inoculation of 10^7 cells producing a solid tumour easily visible by eye within 5 days which continued to grow steadily until approximately 2 cm in diameter at 21 days. If 10^5 cells were used, 3/4 mice developed tumours. Inoculation of less than 10^5 cells produced no tumour. With regard to the other two cell lines, 1.5×10^6 cells of 367 produced visible tumours in 3 weeks and less than 1.5×10^5 cells produced no tumour, while 10^6 111 cells produced visible tumours in 2–3 weeks. Metastases were never seen macroscopically. In addition to the three cell lines, various epithelial cell strains derived from canine mammary carcinomas were also tested in nude mice, plus a fibroblastic strain. None induced tumours when injected subcutaneously at 10^7 cells per mouse. Hamsters, five male and six female, injected subcutaneously on the day of birth with 2×10^6 REM 134 cells did not develop tumours.

Subcutaneous inoculations of REM 134 cells in nude mice produced solid-type carcinoma formations as sub-epidermal or dermal nodules. There was extensive cellular pleomorphism in tumours with varying cell size and indistinct cell outlines. Some cells stained more deeply than others and some pyknotic cells were seen. Nuclei were often variable in size and bizarre shapes were seen; mitoses were commonly observed. A striking feature was the presence of squamous metaplasia in many parts of the tumour nodules (Fig. 1). Central areas of larger tumours were sometimes necrotic, with pyknotic cells sandwiched between necrotic and viable zones. The borders of neoplasms were irregular but well-demarcated; however, there was no well-recognisable complete compression capsule. In some sites the tumour cells infiltrated into surrounding muscle or connective tissue. Part of this effect may have been related to the relative depth of initial subcutaneous inoculation of cells. In some tumours there was evidence of satellite tumour nodules in connective or muscle tissue and, not uncommonly, tumour cells impinged on capillaries at the periphery of tumour foci. The cells were equally undifferentiated in both female and male nude mice.

In some mice inoculated with REM 134 cells the tumours invaded into the lumbar musculature and at autopsy were found to have tumour masses encroaching on the peritoneal cavity. In none of these mice, howver, was there evidence of spread of tumour to abdominal or other body organs, either continuously or by metastasis.

Tumours induced by 111 cells were solid aggregations of epithelial cells (Fig. 2). Although tumour foci were well-demarcated there was only thin and incomplete compression capsule formation. The tumour cells did not appear to infiltrate the surrounding tissues but the peripheral connective tissue was well-vascularised. In contrast to REM 134, there was no evidence of squamous metaplasia, although areas of central necrosis were a regular feature with the same pyknotic cell interfaces as described above. Cellular pleomorphism was observed but, compared with REM 134-induced tumours, cells were more uniform. Typically, they had indistinct cell borders with large pale, but regularly ovoid, nuclei. Mitotic figures were common. Male and female tumours were similar.

Murine tumours produced by inoculation of 367 cells contrasted markedly with REM 134 and 111 tumours (Fig. 3a). Tumour architecture was typically well-organised tubular or papillary adenocarcinoma (Fig. 3b). This appearance, as in the case of REM 134 and 111 tumours, resembled closely the original canine tumours. Larger tumours exhibited central foci of necrosis and aggregations of pyknotic cells were present at the interface between necrotic and viable tumour zones. Sometimes macrophage-like cells were seen. Tumour borders were fairly well circumscribed by compression capsules but the tumours induced in male mice had less prominent encapsulation than in females. Tumour cells had large pale-staining ovoid nuclei with sparse cytoplasm and indistinct cell membranes.

Mitoses were less commonly observed than in REM 134 and 111 tumours. An interesting feature was the fact that 367 tumours in male mice were apparantly more differentiated and organised as tubular elements than in female mice.

Frozen sections of tumours induced by REM 134, 367 and 111 were shown to contain fibronectin as measured by indirect immunofluorescence (Table 1), despite the cells not synthesising it in detectable amounts *in vitro* [1]. Fibronectin was also present in three canine mammary carcinomas tested. Serial tumour passage from one mouse to another was carried out three times using tumours induced by REM 134, 367 and 111 cells which were excised, disaggregated with collagenase/dispase and reinjected subcutaneously. In each case the resulting tumours were the same as regards gross morphology and histopathology as the originals. Cell cultures derived from the disaggregated

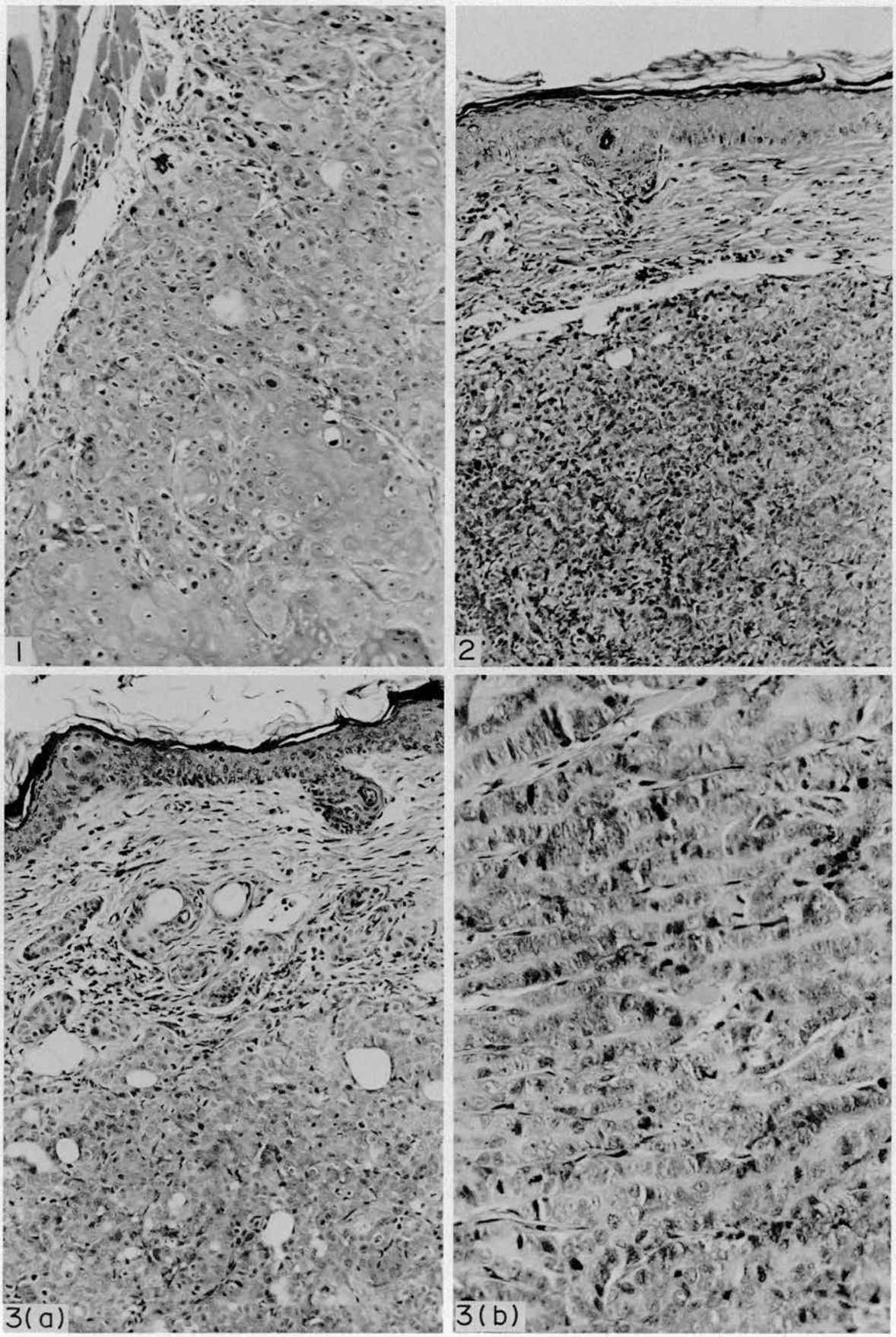

Fig. 1. *Histopathology of tumour induced by REM* 134 *cells in athymic nude mouse* (×160; *H and E*).
Fig. 2. *Histopathology of tumour induced by* 111 *cells in athymic nude mouse* (×160; *H and E*).
Fig. 3. *Histopathology of tumour induced by* 367 *cells in athymic nude mouse* (*a*; ×160; *H and E*), *showing* (*b*) *the well-organised tubular adenocarcinoma structure* (×320).

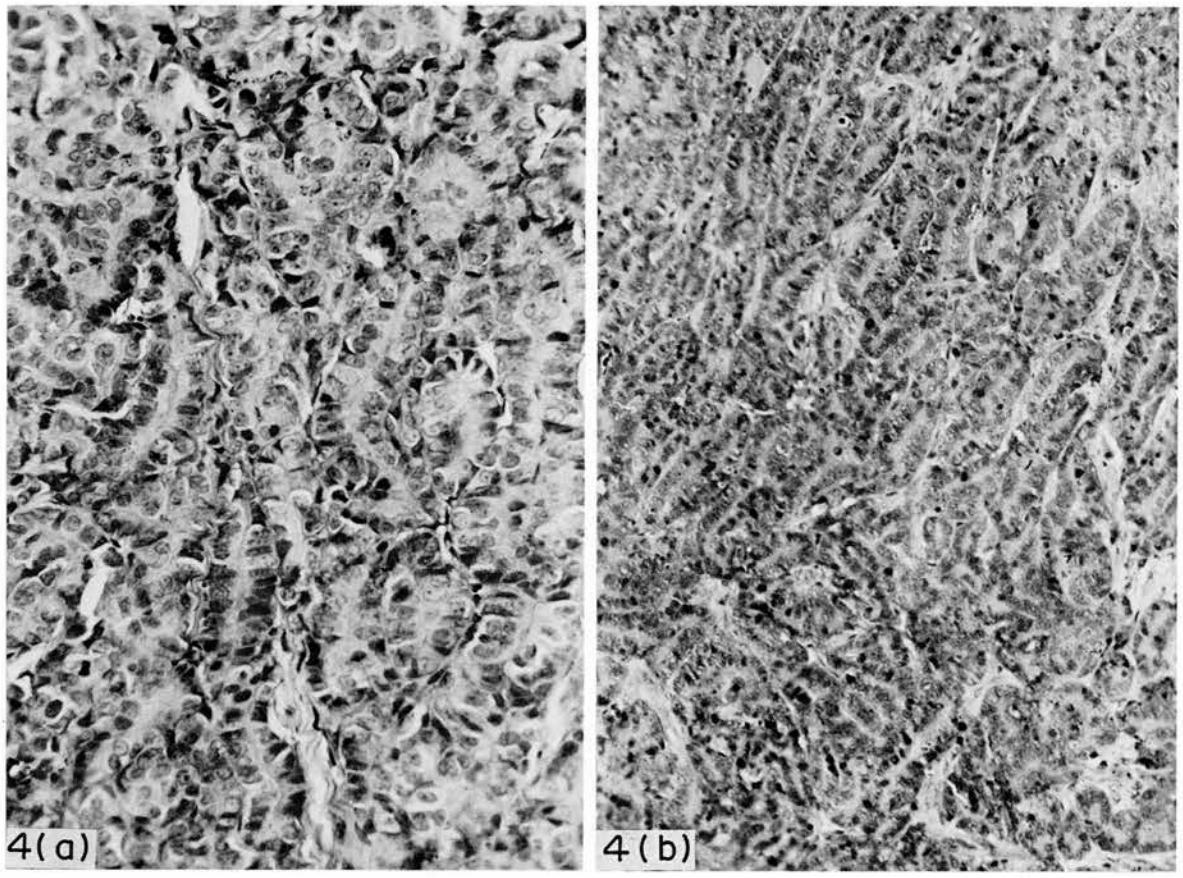

Fig. 4. Histopathology of tumours induced by two clones of 367 cells in athymic nude mice (a, ×320; b, ×160; both H and E).

Table 1. *Presence of fibronectin as measured by indirect immunofluorescence on sections of tumours*

	Dilutions of antiserum		
	1/20	1/100	1/250
Mouse tumour induced by REM 134 cells	+++	++	-
Mouse tumour induced by 367 cells	++	-	-
Mouse tumour induced by 111 cells	+++	++	-
Mammary carcinoma A	+++	++	+
Mammary carcinoma B	++	++	+
Mammary carcinoma C	++	+	-

+++, ++, + = degrees of immunofluorescence; - = no immunofluorescence.

tumours yielded monolayers with the same properties as the parent cells.

It has been reported that injection of MCF-7 mammary carcinoma cells in different sites in athymic mice may lead to development of tumours with different rates of proliferation and invasiveness [5]. Thus 1.5×10^6 REM 134 cells were injected intracranially and 5×10^5 intrapleurally. A tumour was visible in the first case on the head of the mouse within 4 days. It was killed after 11 days, when the tumour measured 8 mm. Histological examination showed extensive invasion of tumour cells around and below the meninges into the brain and cranially into the ethmoid and frontal regions of the head. The neoplasia was anaplastic carcinoma with squamous metaplastic foci, as observed in the original canine tumour and the subcutaneous murine lesions described above. Although there was intra-cerebral growth of tumour cells, there was no evidence of metastases elsewhere in the mouse.

After intrapleural injection the mice were kept for 1 month and then killed. In the case of REM 134 there was copious pleural fluid when the mouse was opened. This was cultured *in vitro* and within 48 hr there was a good growth of cells with typical REM 134 morphology. Macroscopically, mice inoculated intrapleurally showed friable white nodular growths on the mediastinum and parietal pleura, and frequently on the diaphragm. The neoplasia appeared to originate commonly on the mediastinum. Gross sectioning of lung tissue showed no obvious tumour involvement of the parenchyma or pleural surfaces.

Histologically, tumour tissue showed typical REM 134 morphology with a marked degree of pleomorphism, high mitotic index and bizarre nuclei, together with early squamous metaplasia. Where parietal pleural nidation was observed there was neoplastic infiltration of adjacent musculature. Tumour growth was confined to the chest cavity macroscopically, and histological examination of other selected organs showed no tumour metastases.

Intrapleural inoculation of 367 and 111 cells resulted in similar localised tumour growth patterns but no evidence of metastatic disease was observed. In each case the tumours induced were characteristic of the original subcutaneous lesions.

REM 134 and 367 cells were cultured in semi-solid agar and several clones grown up individually [1]. Four REM 134 clones (2×10^5 cells of each) and three 367 (2×10^6 cells of each) were then tested for their tumorigenicity in nude mice by subcutaneous injection. In all cases tumours were induced but their rate of growth varied, a property which correlated with their *in vitro* rate of growth. Histologically, the tumours induced in mice by different clones of REM 134 were identical and similar in type to the initial subcutaneous uncloned mouse tumours; for example, the histopathology of two tumours induced by two such clones is depicted in Fig. 4(a and b).

There was some preliminary evidence to suggest that REM 134 cells induced a slower rate of tumour growth in male mice than in females [2]. In addition, a response to oestrogen had been noted with these cells *in vitro*, a property not shared by the 367 and 111 cell lines [1]. Thus three male and three female mice of the same age were injected subcutaneously with 5×10^5 REM 134 and 367 and 10^6 111 cells. The mice were weighed weekly and the dimensions of any tumour formed measured.

The area of each tumour was calculated by multiplying the diameter measurements together. Height was not included as it was only possible to measure this dimension when the tumour was large. Figure 5(a, b and c) depicts the results obtained for male and female mice injected with REM 134, 367 and 111 respectively. Regression coeffieients were calculated using the area of the tumour as the dependent variable and days post-injection as the independent. All regressions were significant. Weighted T tests were performed with these coefficients as independent variables weighted by the reciprocal of the estimated variance of each coefficient. The weighted means for the male mice were tested against those of the female for each of the three cell lines, giving the results of $T_4 = 1.6$ for REM 134, $T_4 = 0.33$ for 111 and $T_4 = 0.54$ for 367. None of these was significant.

The regressions were repeated using various log transformations (log area vs day, log area vs log day, area vs log day) which produced improved fits for some mice, notably two females injected with REM 134, although for most mice the

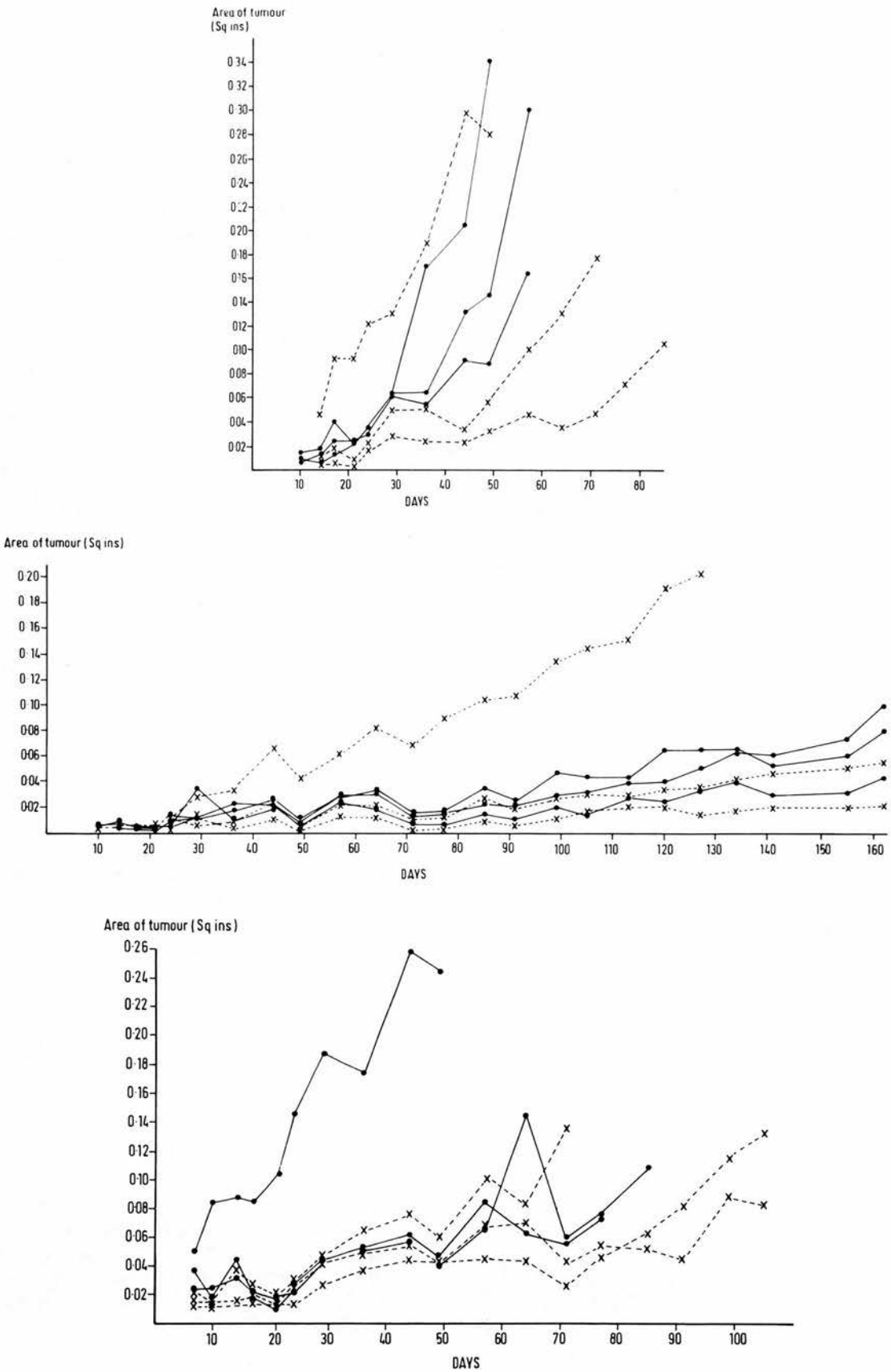

Fig. 5. Rate of growth of tumours in male (×-----×) and female (●——●) athymic nude mice induced by (a) REM 134, (b) 367 and (c) 111 cells.

original regression gave the best fit. Thus the weighted T test was calculated on the transformed (log area) data for REM 134, giving $T_4 = 2.05$, $0.1 < P < 0.2$. This was considered of marginal significance only.

Calculation of the regression coefficients from an analysis of weight of mice on days post-infection did not suggest any relationship with the growth rate of tumours (data not shown).

DISCUSSION

Cell suspensions from three canine mammary carcinoma cell lines were found to induce tumours when inoculated subcutaneously in athymic nude mice. These tumours were similar in general histological type to their canine originals, and could be passaged as cell suspensions from one mouse to another directly or as *in vitro* tissue cultures.

An interesting feature of the lines was that 367 appeared to be less aggressive, with a greater number of cells required to induce tumour growth subcutaneously. Morphologically, 367 tumours were better differentiated tubular or papillary carcinomas, as opposed to the anaplastic epithelial neoplasms induced by REM 134 and 111 cells. In all the mice inoculated, however, there was no gross or microscopic evidence of metastatic disease with any of the tumour types. Some tumours induced by REM 134 showed early infiltration of adjacent musculature but, in common with all the subcutaneous neoplasms, the cells were well-circumscribed by a compression pseudo-capsule. In some cases with 134 and 111 cells there was evidence of 'pushing' expansion of tumours but capillaries at the edge of tumours showed no convincing evidence of tumour cells within their lumens, as might have been expected in early metastasis. One possible explanation for apparent tumour-to-muscle infiltration may be related to site of original cell inoculation such as inadvertently seeding cells into muscular tissue rather than subdermal areolar connective tissue. The lack of metastasis agrees with most other studies [3, 6, 7], although Ozzello and Sordat report that some human mammary cell lines do metastasize at variable frequency [8]. It might be worthwhile allowing tumours to remain *'in situ'* for longer periods or to 'trigger' any metastatic potential by use of agents such as collagenase.

Attempts were made to induce metastatic disease by inoculating cells intracranially in the case of REM 134, and intrapleurally for all three lines. Florrid tumour growth occurred but there was still no induction of metastatic disease.

Examination of clones of 134 and 367 prepared by growth of colonies in semi-solid agar showed differences in rates of growth in mice but histologically the tumours induced had no well-defined differences from the originals. Ultrastructural examinations have so far yielded no further explanation for the different growth rates.

Initial studies [1] showed that the cell lines exhibit no hormone receptors when subjected to standard receptor assay; this is in contrast to the findings of Thomas *et al.* [4], who reported the presence of oestrogen receptors in four solid cell lines which grew in nude mice. *In vitro* studies for REM 134 cells indicated an increased growth rate in the presence of oestrogen and luteotropic hormone [1], and some preliminary work showed that there may be a difference in growth rate of tumours induced by REM 134 cells in male and female mice [2]. However, the present results, using larger numbers of mice and statistical analysis, showed that there was no significant difference in growth rate between males and females. It is possible that any hormone receptors present on the original canine tumours from which the cell lines were derived may have been masked or lost during passage, and the apparent response of REM 134 cells *in vitro* due to some other factor. To clarify this matter, we are currently testing the effect of tamoxifen, an anti-oestrogenic agent, on growth rate of REM 134 cells *in vitro* and tumour induction in male and female nude mice.

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THE EFFECT OF TAMOXIFEN ON TUMOURS INDUCED BY CELLS FROM A CANINE MAMMARY CARCINOMA CELL LINE IN ATHYMIC NUDE MICE

Mary Norval*, R.W. Else+ and Jean Maingay*.

*Department of Bacteriology, University of Edinburgh Medical School Teviot Place, Edinburgh

+Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh

Running title: Effect of tamoxifen on canine mammary tumours induced in athymic mice.

SUMMARY

Tumours were induced in CBA athymic nude mice by subcutaneous injection of REM 134 cells. These cells were from a continuous line derived from a canine mammary carcinoma and have no detectable oestrogen receptors. In vitro, the anti-oestrogen tamoxifen was growth inhibitory at a concentration of 10^{-6}M and adding 10^{-8}M oestradiol-17β did not reverse this effect. The relative rate of growth of the tumours induced by the cultured cells was the same in male and female mice. Oral tamoxifen at a dose of 1 mg/mouse/week suppressed the growth rate significantly and to the same extent in male and female mice; subcutaneous tamoxifen at 2 mg/mouse/week had no significant effect. These results argue against tamoxifen acting solely as an antagonist for oestrogen.

INTRODUCTION

Tamoxifen, one of the synthetic triphenyl-ethylene compounds, has been used as a specific anti-oestrogen for the past 10 years in the treatment of both locally advanced and disseminated breast cancer in post-menopausal women (Mourisden et al,1979). More recently a trial was undertaken to study the effect of tamoxifen in all women undergoing mastectomy for early breast cancer (Nolvadex Adjuvant Trial Organization, 1983). A significantly prolonged disease-free interval and survival was found in both pre- and post-menopausal women and in cases with and without lymph node metastasis. It is thought that tamoxifen exhibits its anti-tumour activity through binding to the specific oestrogen receptor protein (ER) in the cytoplasm of some breast tumour cells (Furr et al, 1979)although the exact mode of action is unknown. Originally in several surveys, mostly involving small numbers of patients, it was found that 47% of ER-negative tumours responded completely or partially to tamoxifen treatment (reviewed in Patterson et al, 1982). This latter figure casts some doubts on the ER hypothesis, and in the Nolvadex Trial mentioned already, no correlation was found between ER status and response to tamoxifen treatment.

Tamoxifen studies in various animal species show that its effect varies from species to species; in mice it is thought to be a full oestrogen agonist (reviewed in Patterson, 1981).

In dogs there is little recorded information as to the action of tamoxifen in normal or tumour-bearing bitches. In a pilot study carried out by one of the authors (RWE, unpublished results) the use of oral tamoxifen was found to be useful in bitches with ER-positive tumours but a major disadvantage was the production of endometrial

hyperplasia with vaginal discharge in entire females. Another study on the use of tamoxifen for post-mastectomy therapy in bitches is currently in progress in South Wales (Pierrepoint, personal communication).

A cell line, REM 134, derived from a spontaneous primary canine mammary carcinoma has been described (Else, Norval and Neill, 1982). Its in vitro properties have been investigated (Norval, Maingay and Else, 1984a) and its ability to induce tumours in athymic nude mice (Norval, Maingay and Else, 1984b). The cells had no demonstrable oestrogen receptors, and the rate of tumour growth did not differ between male and female nude mice. An investigation was undertaken to ascertain the effect of tamoxifen and the mode of administration on tumour growth in this system in the hope of elucidating the mechanism of action of tamoxifen in vivo.

MATERIALS AND METHODS

Mice

CBA athymic nude mice, 4-6 weeks old, were purchased from the Clinical Research Centre, Harrow. The mice were kept in an isolator with autoclaved bedding, filtered air, sterile food and acidified water (pH 2.8). Cells were injected subcutaneously into the centre of the back. Tumour diameters in 2 directions were measured using vernier callipers. The mice were weighed weekly. When the tumours became necrotic or reached around 2 cms in diameter, the mice were killed by cervical dislocation. Tumour tissues were weighed and fixed for histopathology in 10% buffered formalin, together with selected organs prior to processing as paraffin and resin block sections.

Tamoxifen

This was a gift from ICI Ltd., Alderley Park. Just before use, it was weighed and suspended with Vortex mixing in 10% w/v sucrose in 0.01M phosphate buffered saline, pH 7.2 (PBS) at a concentration of 0.5, 1 or 2 mg/0.1ml. 0.1ml was injected into the mice subcutaneously or given orally, by introduction into the back of the throat using a small canula, once per week.

Cells

REM 134 cell line was derived, cultured and maintained as already described (Else, Norval and Neill, 1982). For inoculation into mice, the cells were prepared as outlined in Norval, Maingay and Else, 1984b. To measure the effect of tamoxifen *in vitro*, 5×10^3 cells were inoculated into wells of 96-well microtitre plates (Nunc) in 0.2 ml medium containing 1% foetal calf serum. Tamoxifen, dissolved in ethanol at a concentration of 1 mg/ml, then diluted in medium, was added to give final concentrations of 10^{-6}, 10^{-7} and

10^{-8}M. One μC_i ^{3}H-thymidine (Amersham) was added to 12 wells of each concentration of tamoxifen and 12 wells containing no tamoxifen. The plates were incubated at 37°C in 5% CO_2 humidified atmosphere. Twenty four-hours later the cells were harvested using an Ilacon Harvester on to glass fibre discs which were washed in water, cold 10% trichloroacetic acid, and ethanol before being counted in PPO-toluene scintillator in a Packard scintillator counter. ^{3}H-thymidine was added to further sets of wells after 1,2,3 and 4 days and the cells harvested after 24 hours. In addition, 10^{-8}M oestradiol-17β was added to 1 set of wells in the presence of 10^{-6}M tamoxifen. The mean of the 12 counts was taken for each time point, the standard error of the mean being within 10% of the mean in all cases.

RESULTS

The response of REM 134 cells *in vitro* to tamoxifen was first ascertained in case the cells were resistant to the drug. It was found that growth of REM 134 cells was inhibited by 10^{-6}M tamoxifen as measured by the uptake of ^{3}H-thymidine. At 10^{-7}M growth was slowed, and at lower concentrations no difference was found when compared to cultures without tamoxifen. Adding 10^{-8}M oestrogen-17β did not alter the response to 10^{-6}M tamoxifen. These results are shown in Fig.1. It was not possible to measure cell growth by counting numbers of viable cells as the REM 134 cells clumped together after removal from the culture vessel, even in the presence of 0.1% trypsin.

From previous experiments it was already known that REM 134 cells at numbers over 10^{5} induced subcutaneous solid-type carcinoma tumours in CBA nude mice and the rate of tumour growth in 3 male and 3 female mice did not differ significantly (Norval, Maingay and Else, 1984b).

In the first experiment, 11 male and 10 female CBA nude mice were injected subcutaneously with 5×10^{5} REM 134 cells. Five days later 1 group of 3 or 4 mice of each sex was treated orally with 0.5 mg tamoxifen, another group with 1 mg and the third was given no tamoxifen. Tamoxifen was given weekly thereafter, to appropriate groups, and tumour dimensions were measured twice weekly starting on day 20 and finishing on day 76. The results are shown in Fig. 2a, b and c, the area of the tumours being calculated by multiplying the two diameter measurements together.

From inspection of the graphs, it may be seen that growth rate of tumours was depressed in the group of mice given 1 mg tamoxifen weekly by the oral route while 0.5 mg tamoxifen had an equivocal effect.

There was rather a widespread response by the group of control mice which made statistical analysis of these results unsatisfactory. It was possible to determine the effect of sex on the growth rate of tumours using regression coefficients calculated with days post-injection as an independent variable and area of tumour as a dependent variable and performing an analysis of variance. No significant difference was found between the sexes. The weights of the male and female mice in the tamoxifen groups were the same as in the control group throughout the period of the experiment (data not shown).

A second experiment was completed in which 12 CBA male and 12 female mice were injected subcutaneously with 2.5×10^6 REM 134 cells. Starting 4 days later, 1 group of 4 mice of each sex was treated orally with 1 mg tamoxifen per week, a second with 2 mg injected subcutaneously, and a third was given no tamoxifen. Tumour measurements were started on day 11 and then weekly till day 36. The results are depicted in Table 1.

Coefficients were calculated from individual regressions of days post-injection against area of tumour (with zero intercept). They were used as independent variables in analyses of variance with 2 grouping factors, treatment and sex. The first analysis compared the control group and 1 mg oral dose group and the second the control group and 2 mg subcutaneous group. The results show that sex was not a factor in determining growth rate of tumours, as had already been found in the first experiment. Analysis of the control group and 1 mg oral dose group show a difference just below significance between the means of the 2 groups (F value 3.97; probability 0.087). There was no significant difference between the means of the control group

and the 2 mg subcutaneous dose group (F value 0.51; probability 0.49).

Thus, bearing in mind the difficulty in finding a regression formula which adequately described the growth of tumours in all the mice and the rather variable growth responses in the control group, a dose of 1 mg tamoxifen given orally was considered to reduce the growth rate of the tumours to a significant extent. Weights of mice in both tamoxifen groups did not differ from the control mice.

Histopathological examination was made of haematoxylin and eosin stained tumour sections from mice treated with tamoxifen and compared with sections from control tumour-bearing mice. In addition, selected organ sections (liver, kidney, spleen, lungs, brains, ovaries and uterus) were compared. There was variation in degrees of central necrosis in all groups depending on the size of the tumour mass, but there was no obvious difference in tumour morphology or body distribution between tamoxifen treated groups and controls. Similarly, siting of tumour borders below the epidermis relative to the superficial dermal muscle bundles varied amongst individuals within each treatment group but not between groups. This variation was probably directly attributable to the site of the original inoculation.

In none of the treatment groups was inflammatory cellular infiltration a feature. Tumour borders were usually well-defined by a compression pseudo-capsule except in some large control tumours which had become ulcerated. There was no evidence of capillary or lymphatic permeation by tumour cells in the area surrounding tumours, and no change in vascularity of tumours in treated and control groups. A difference in tumour cell morphology was not noticed between groups. Cellular pleomorphism was equally marked in all

groups and the mitiotic index of tumours was similar.

Examination of sections of organs showed no evidence of metastatic tumours in either control or treated mice. Some variation in ovarian and uterine histology was seen in different treatment groups; these results are to be reported elsewhere.

DISCUSSION

In recent months it has been a matter of some debate whether tamoxifen acts solely through binding to cytoplasmic oestrogen receptors or whether it may have additional targets (Baum, 1984). This is of importance when considering treatment of patients who have breast cancer and who have differing menopausal, nodal and ER status. Apart from acting as an oestrogen antagonist, several other theories have been advanced to explain the mechanism of action of tamoxifen, especially in women who are ER negative. In the first place, an anti-oestrogen binding protein has been described in human tissues which is larger than ER and ubiquitous, unlike ER (Sutherland et al, 1980). This protein binds tamoxifen with high affinity, while oestradiol competes weakly for the same site. A similar anti-oestrogen binding site distinct from the oestrogen receptor has also been identified in rat tissue (Sudo,Monsma and Katzenellenbogen, 1983). It is present in the microsomal fraction and distributed widely, its level not paralleling that of the oestrogen receptor. Secondly it is possible that growth factor activity may be altered by tamoxifen binding, as suggested by Baum (1984). The cell membrane may be changed, thus influencing the ability of the cells to respond to other hormones like insulin, pituitary hormone, growth hormone and, perhaps most significantly, prostaglandins. Tamoxifen inhibits synthesis of prostaglandins *in vitro* (Ritchie, 1978) and less prostaglandin may inhibit growth of tumour cells *in vivo* while increasing the immune response.

Only a few reports on the oestrogen receptor status of primary mammary tumours in dogs have been published (Hamilton, Else and Forshaw, 1977; D'Arville and Pierrepoint, 1979; MacEwen, Patnaik,

Harvey et al, 1982). These studies have confirmed that between 50-60 per cent of primary tumours are ER-positive and that the majority of such tumours are histologically benign although the correlation between ER-positivity, tumour type and survival is not clear-cut. Virtually nothing is known concerning the effects and mode of action of tamoxifen in tumour-bearer or post-mastectomy bitches.

In this study, a canine mammary carcinoma cell line REM 134, which has been well characterized has been used to examine the effect of tamoxifen on tumour growth in athymic nude mice. Addition of tamoxifen at a concentration of 10^{-7}M to REM 134 cells cultured *in vitro* had a growth inhibitory effect. At 10^{-6}M it was cytotoxic and this was not reversed by oestradiol-17β. Human mammary carcinoma cell line, MCF-7, has been shown to respond to tamoxifen in several ways. At low concentrations there was evidence of oestrogen agonism but at higher concentrations tamoxifen suppressed progesterone receptor synthesis and cell growth and behaved as an oestrogen antagonist (Horwitz, Koseki and McGuire, 1978). In this respect our observation that tamoxifen in bitches may produce an exaggerated progesterone-like effect culminating in endometrial hyperplasia is relevant. Other groups have also showed suppression of MCF-7 cell growth, reduction of plasminogen activator activity and suppression of progesterone receptor levels (Coezy, Borgna and Rochefort, 1982; Katzenellanbogen et al, 1984). MCF-7 cells contain ER, but REM 134 cells have no detectable ER or progesterone receptors. While it is possible that these receptors may be present at a level below the sensitivity limit of the assay system, it seems more probable that tamoxifen is acting by some other mechanism in the REM 134 cells.

In vivo REM 134 cells induced tumours when injected

subcutaneously into athymic nude mice. These tumours were solid type carcinomas formed as sub-epidermal or dermal nodules, were undifferentiated and did not metastasise (Norval, Maingay and Else, 1984b). No difference in growth rate was found between male and female mice, thus indicating that oestrogen receptors are probably not important in this system. Tamoxifen given orally at a dose of 1 mg/week reduced the growth rate of tumours; 0.5 mg/week had no effect, nor did 2 mg/week administered by subcutaneous injection. Both sexes gave the same results.

Thus we have shown that oral tamoxifen inhibits growth of mammary tumour cells in athymic nude mice, and that the mechanism of action is unlikely to be by tamoxifen acting as an oestrogen antagonist. The route of administration of the drug appears to be important and subcutaneous injection presumably does not allow circulation to organs or to the tumour site where tamoxifen exerts its effect. These results may have some significance for the treatment of women with breast cancer and lend some further support to the concept that tamoxifen may act both in vivo and in vitro by more than one mechanism.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust. We thank Mrs G. McConnell, Pre-Clinical Studies, R(D)SVS, for statistical work.

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TABLE 1. Area of tumours ($cm^2 \times 10^{-2}$) induced by REM 134 cells in athymic mice treated with tamoxifen.

		Days post-injection				
		11	18	25	32	36
No tamoxifen	Male mice	23 29 22 21	64 69 38 43	106 109 75 76	171 161 165 164	204 202 169 198
	Female mice	18 22 22 32	43 61 49 78	48 88 80 138	74 103 136 267	130 122 156 440
1 mg tamoxifen weekly, given orally	Male mice	17 17 14 22	37 48 28 37	61 81 78	106 95 119	119 110 116
	Female mice	22 14 12 18	41 25 28 40	97 25 50 67	147 44 62 106	178 44 77 110
2 mg tamoxifen weekly, given sub-cutaneously	Male mice	18 10 19 23	64 24 49 57	99 33 94 82	154 58 126 139	156 59 167 137
	Female mice	18 18 15 14	62 46 48 56	102 96 52 81	151 193 100 121	172 233 133 126

FIGURE LEGENDS

Fig 1. Mean counts per minute incorporated into REM 134 cells during incubation in the presence of 10^{-6}M tamoxifen (●——●), 10^{-6}M tamoxifen and 10^{-8}M oestradiol-17β (o——o), 10^{-7}M tamoxifen (▲——▲), 10^{-8}M tamoxifen (△——△), and no tamoxifen (■——■). ^{3}H-thymidine was added on day 0, 1, 2, 3 and 4 and cells were harvested 24 hours later.

Fig 2. Area of tumour at various times after subcutaneous injection with REM 134 cells; (a) no tamoxifen given, (b) 0.5 mg tamoxifen weekly given orally, (c) 1 mg tamoxifen weekly given orally.

(male mice o——o, female mice ●——●).

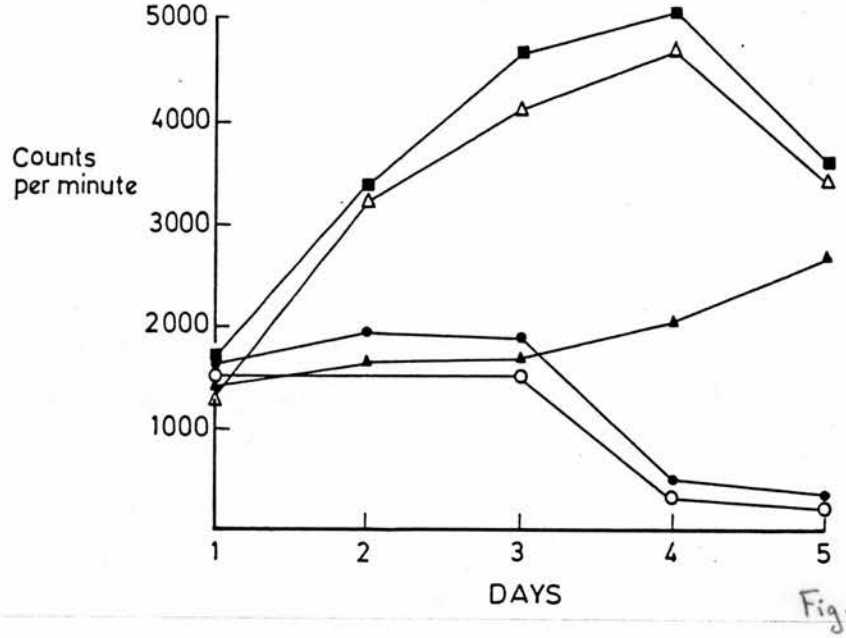
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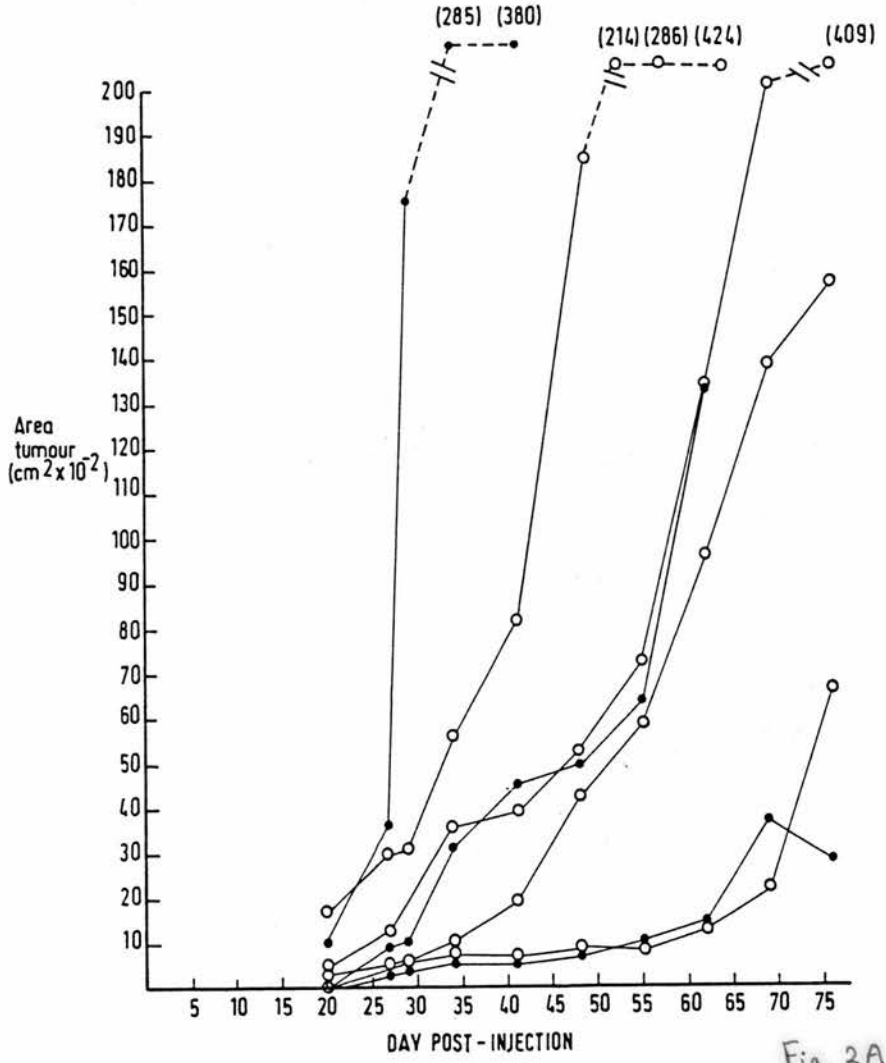
Fig. 1.


(285) (380)
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200
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Area tumour (cm 2 x 10-2)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
DAY POST-INJECTION

Fig. 2A.

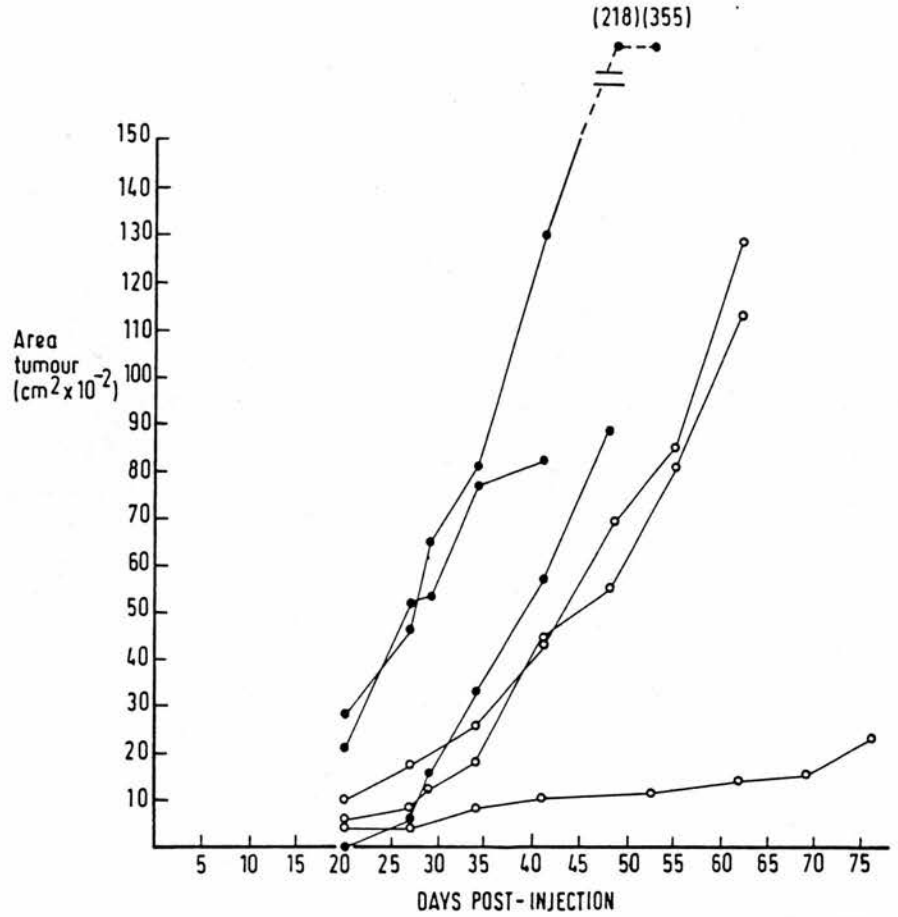

(218)(355)
Area tumour ($cm^2 \times 10^{-2}$)
DAYS POST-INJECTION

Fig 2b.

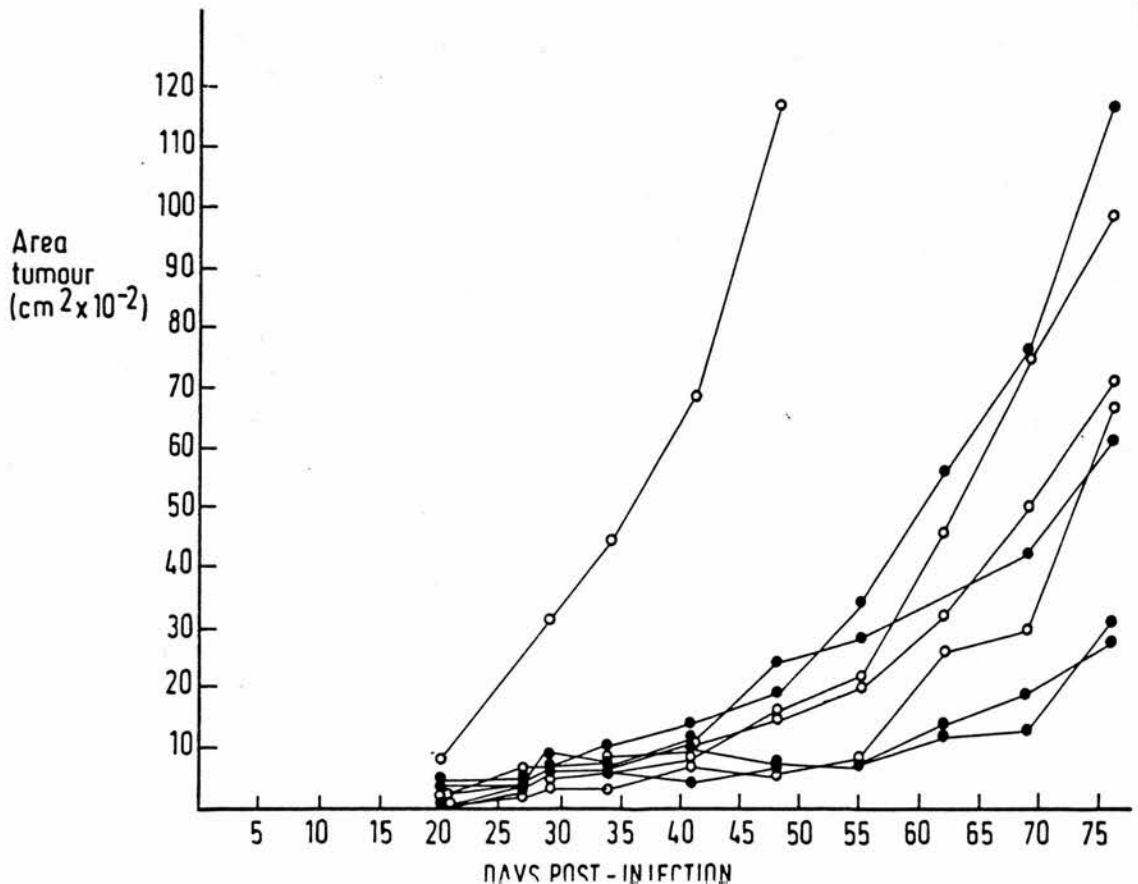

Area tumour ($cm^2 \times 10^{-2}$)
DAYS POST-INJECTION

Fig 2c.

Research in Veterinary Science 1985, 39, 157–164

Characteristics of a feline mammary carcinoma cell line

M. NORVAL, J. MAINGAY, *Department of Bacteriology, University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG*, R. W. ELSE, *Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh QEH9 1QH*

The establishment of an epithelial cell line, JM, from a feline mammary adenocarcinoma is described. In vitro, the morphological and cultural properties of these cells, their surface features such as lectin binding and their response to hormones were ascertained. In vivo, they are tumorigenic in athymic nude mice inducing tumour nodules composed of epithelia-like cells.

STUDIES have been made on cell lines derived from mammary carcinomas in several species, such as mouse (Owens and Hackett 1972), dog (Owen et al 1977) and man (Soule et al 1973). Such cells have been investigated morphologically and their response to various hormones, hormone receptor status and tumorigenicity in experimental animals assayed. These cells may provide 'in vitro' models for testing steroid hormone and other related drugs used in treatment of mammary carcinomas.

With regard to the cat, Dorn et al (1968) stated that mammary tumours were the third commonest of all feline neoplasms. In a comparative review of mammary neoplasia, Hamilton (1974) found that these tumours occurred in domestic cats half as often as in bitches. Most female cats, in Britain at least, are subjected to ovariohysterectomy at, or soon after, puberty (five to six months old), thereby minimising any ovarian influence on mammary tissue. However, Hamilton et al (1976) reported that the majority of feline mammary carcinomas showed no demonstrable oestrogen receptor activity.

Nearly all mammary tumours in the cat are carcinomas (80 per cent, R W. Else, personal observations) and the most common type is the adenocarcinoma (Hayden and Neilsen 1971). The carcinomas resemble those occurring in women in being highly aggressive. They rapidly metastasise to multiple visceral sites and, although bone metastases are unusual (Kas et al 1970), Weijer et al (1972) have suggested that feline mammary carcinoma is a valuable comparative intermediate model between mouse and man. Little recent work, however, has been done to investigate further the cat model, particularly with regard to studies of metastasis.

In this paper, a description is given of the origin of a cell line called JM which is derived from a feline mammary carcinoma. Its morphological properties, surface features, response to hormones and tumorigenicity in athymic nude mice are characterised.

Material and methods

Clinical material and primary culture

Pieces of mammary tumours were obtained from cats undergoing euthanasia or surgery. Within two hours, small fragments were disaggregated in collagenase/dispase (Boehringer) for one to two hours at 37°C. The single cells were washed and suspended in tissue culture medium, Eagle's minimal essential medium supplemented with 100 iu ml^{-1} penicillin, 200 μg ml^{-1} streptomycin, 50 iu ml^{-1} fungizone, 100 μg ml^{-1} gentamicin and 10 per cent fetal calf serum. Incubation was carried out at 37°C in an atmosphere of 5 per cent carbon dioxide in air using Falcon flasks (Flow) with loose caps. After five days, the medium was changed. If the culture became confluent, the cells were passaged using trypsin-versene. Gentamicin and fungizone were then omitted from the medium.

In vitro properties of JM line

Specimens were prepared for transmission electron microscopy and chromosome spreads made as outlined by Else et al (1982). Doubling time and colony formation in semi-solid agar were also performed as described by Else et al (1982). Fibronectin content, binding of lectins labelled with FITC, induction of retrovirus and response to stimulation by various hormones were all measured as outlined by Norval et al (1984a).

For transfection experiments, high molecular weight DNA was prepared from JM cells using the method of Balmain and Pragnell (1983). Transfection of NIH3T3 and C-127 cells with DNA at concentrations of 30 μg ml^{-1} and 10 μg ml^{-1} was carried out, whereby 1 ml of DNA-calcium chloride coprecipitate

TABLE 1: Binding of FITC-lectins to cell cultures as measured by direct immunofluorescence

	JM cells	Epithelial cells A	Epithelial cells B
PNA	–	–	–
GS-I	+ +	+	+
GS-I + D-gal	–	–	–
BSA-II	+ + +	+ +	+ +
DBA	+ +	–	–
DBA + NAc-D-gal	+	–	–
SBA	+ +	–	–
SBA + D-gal	+	–	–
MPA	–	–	–
MPA + D-gal	–	–	–
RCA	–	+	+
RCA + D-gal	–	–	–
WGA	+ + +	+ + +	+ + +
UEA	–	–	–
UEA + L-fuc	–	–	–
Con A	+ + +	+ +	+ + +
Con A + D-man	+	+	+
Con A + D-glc	+	+	+
LPA	+	–	+

PNA Arachis hypogaea agglutinin
BSA-II Bandeiraea simplicifolia agglutinin
SBA Glycine max agglutinin
RCA Ricinus communis agglutinin
UEA Ulex europaeus agglutinin
GS-I Bandeirea simplicifolia agglutinin
DBA Dolichos biflorus agglutinin
MPA Maclura pomifera agglutinin
WGA Triticum vulgaris agglutinin
Con A Concanavalia ensiformis agglutinin
LPA Limulus polyphemus agglutinin
D-gal D-galactose
L-fuc L-fucose
D-glc D-glucose
D-man D-mannose
NAc-D-gal N-acetyl-D-galactosamine
– No immunofluorescence
+ + +, + + and + Degrees of immunofluorescence

were induced as measured by incorporation of ^{3}H-uridine into particles with the density of 1·16 to 1·18 g ml^{-1}. Recently, many cell cultures derived from tumours have been tested for the presence of oncogenes by the ability of their DNA to transform NIH3T3 cells or C-127 cells (Pulciani et al 1982). However, using DNA purified from JM cells at pass 8, no foci of transformed cells was obtained and thus no evidence of oncogene involvement found.

The athymic nude mouse has proved an excellent experimental model for the induction of tumours and has been used, for example, in studies on the oncogenicity of various canine mammary tumour cell lines (Norval et al 1984b). Cells from JM cultures were inoculated subcutaneously into four-week-old CBA Nu/Nu mice. In three out of three mice inoculation of 3×10^6 cells produced tumours of about 0·4 cm diameter within two weeks which continued to grow steadily until the mice were killed a month later. An inoculum of 10^6 cells led to the formation of a visible tumour in one out of two mice in one month. 10^7 cells of epithelial strain A or B injected subcutaneously into two mice each did not produce visible tumours and subsequent histological examination when the mice were killed two months later was negative for neoplasia.

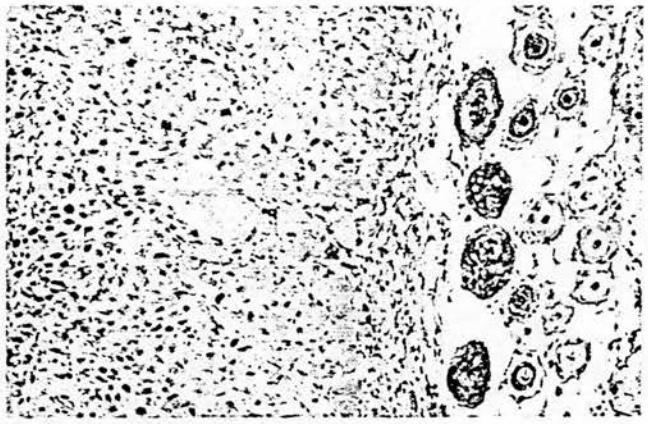
Paraffin wax and plastic resin sections of tumours induced by JM cells showed tumour nodules composed of epithelia-like cells. The borders of the tumours were completely circumscribed by compression pseudocapsules but, in several sites, there was evidence of infiltration of cells into adjacent subdermal musculature (Fig 5a). In general the tumour cells were characteristically anaplastic (Fig 5b), although some sites showed differentiation into tubular elements (Fig 5c) which closely resembled the original feline tumour.

Discussion

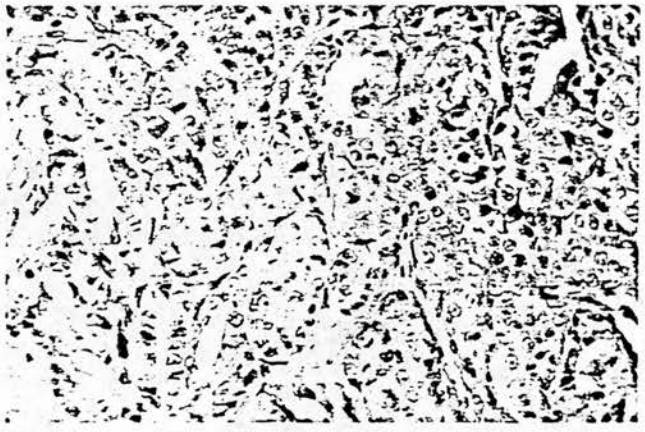
Out of 30 pieces of tumour obtained from feline mammary carcinomas, one yielded cells on culture which had the characteristics of transformed cells. These cells were epithelial in morphology at the light and electron microscope level, and sometimes formed into acinar arrangements as though mimicking their in vivo growth properties. The karyotype did not show any consistent abnormality such as in chromosome number or translocation. The JM cells had the ability to grow in semi-solid agar which has been suggested as a reliable characteristic of most tumorigenic cells (Chen et al 1976). Clones produced by this method had the same morphology as the parent cells.

Lectins labelled with FITC were used to examine the quantity and type of glycoproteins on the surface of JM cells and to compare these with two epithelial cell strains also derived from feline mammary carcinomas. Three differences in binding were detected. The significance of this finding will have to await the isolation of more tumorigenic feline cell lines, and an analysis of antigens on the surface of JM cells, both of which are being undertaken by the authors.

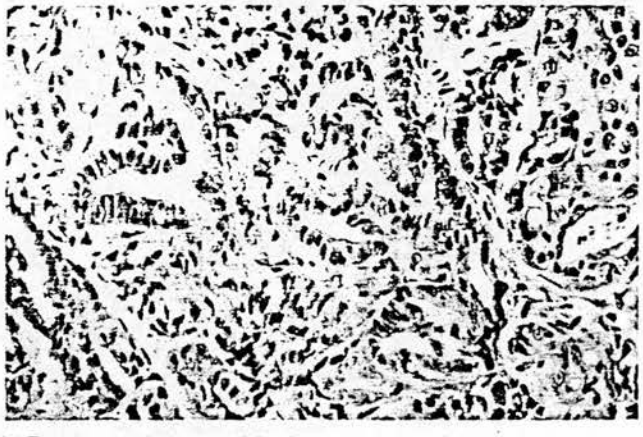
No retrovirus-like particles were observed on electron microscopy or after attempted induction with several agents. In addition no oncogenes were detected using the NIH3T3 and C-127 transformation systems. There have been reports of retroviruses being associated with mammary carcinomas cultured from various species, such as murine (Fine et al 1974), canine (Watrach et al 1978) and human (McGrath et al 1974). However, apart from the first example where murine mammary tumour virus is well established as the aetiological agent of the carcinoma, consistent evidence has not been produced to support the involvement of retroviruses in the other species. In addition, oncogenes have not been demonstrated in DNA from human mammary carcinomas using the



(a) Edge of tumour. H&E × 120



(b) Anaplastic area. H&E × 240



(c) Tubular area of tumour. H&E × 240

FIG 5: Histopathology of tumour induced by JM cells in an athymic nude mouse

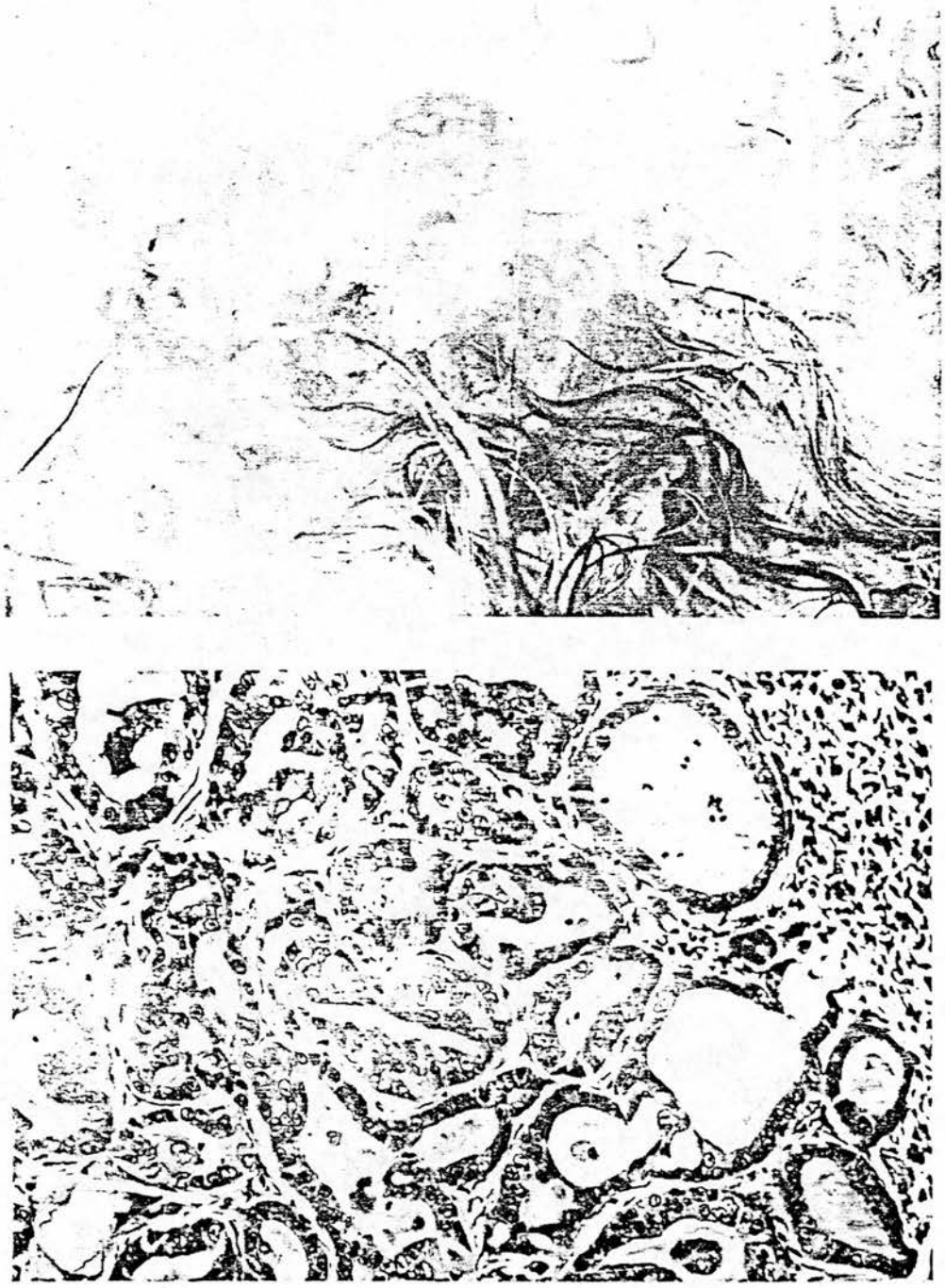


FIG 1: Macroscopic (above) and histological (H&E × 400) (below) appearance of feline mammary tumour from which JM cell line was derived

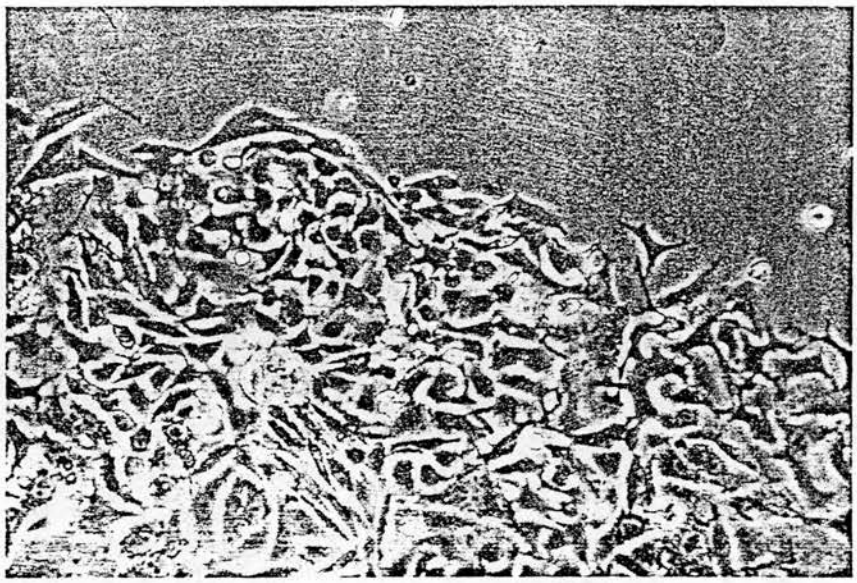


FIG 2: Phase contrast microscopy of JM cells in vitro at passage 6. × 100

was added to 15 ml medium for a 75 cm^2 flask containing 3×10^6 actively growing recipient cells (Campo and Spandidos 1983). DNA prepared from SV-40 was used as a positive control and salmon sperm DNA as a negative control. The cultures were observed for three weeks, changing the medium every third day.

Mice

Female CBA athymic mice, four to six weeks old, were purchased from the Clinical Research Centre, Harrow. They were kept in an isolator with autoclaved bedding, filtered air, sterile food and acidified water (pH 2·8). Injections were carried out subcutaneously. When the tumours reached around 2 cm in diameter, the mice were killed by cervical dislocation. Tumours were excised and part of each fixed for histopathology in 10 per cent buffered formalin, sectioned after embedding in paraffin wax and stained with haematoxylin and eosin.

Results

Tissue from mammary carcinomas of 30 cats undergoing euthanasia or surgery was disaggregated and put into culture. In most cases, there was some cell attachment and growth initially but confluent monolayers did not form and the cells eventually rounded, detaching from the surface. However with four tumours this did not happen; the cells grew well and could be passaged. Two cultures yielded cell strains with epithelioid morphology called A and B, and one with fibroblastic morphology, all of which were used for comparison throughout. The fourth culture yielded the cell line JM which was derived from a highly malignant tubular adenocarcinoma (Fig 1). with fibroblastic morphology, all of which were used for comparison throughout. The fourth culture yielded the cell line JM which was derived from a highly malignant tubular adenocarcinoma (Fig 1). Histologically, the tumour was poorly circumscribed with fibrosis around neoplastic tubular formations and aggregations of macrophages with sparse numbers of lymphocytes and plasma cells were observed frequently at the borders of tumour lobules.

In vitro, JM cells formed distinct epithelial islands (Fig 2). They were passaged over 30 times with no change in morphology. On transmission electron microscopy they showed epithelia-like cell morphology (Fig 3a) with irregular nuclear outlines, some lipid accumulations and vacuole structures (Fig 3b). One clump of cells showed an acinar arrangement with small villiform processes projecting into the lumen (Fig 3c) but this was not a common feature. The cell surfaces exhibited only sparse numbers of short microvilli. No viral particles were observed.

Chromosome preparations were made of JM cells at passage 5. They contained 38 to 42 chromosomes with abnormalities including translocations, dicentrics and fragments. One karyotype, depicted in Fig 4, had 39 chromosomes including one abnormal chromosome and an additional fragment.

The doubling time of JM cells in 10 per cent serum

(a)

(b)

(c)

FIG 3: Transmission electron micrographs of JM cells at passage 6
(a) Typical cells. × 3500
(b) Internal vacuole structure. × 23,100
(c) Acinar arrangement of cells. × 34,400

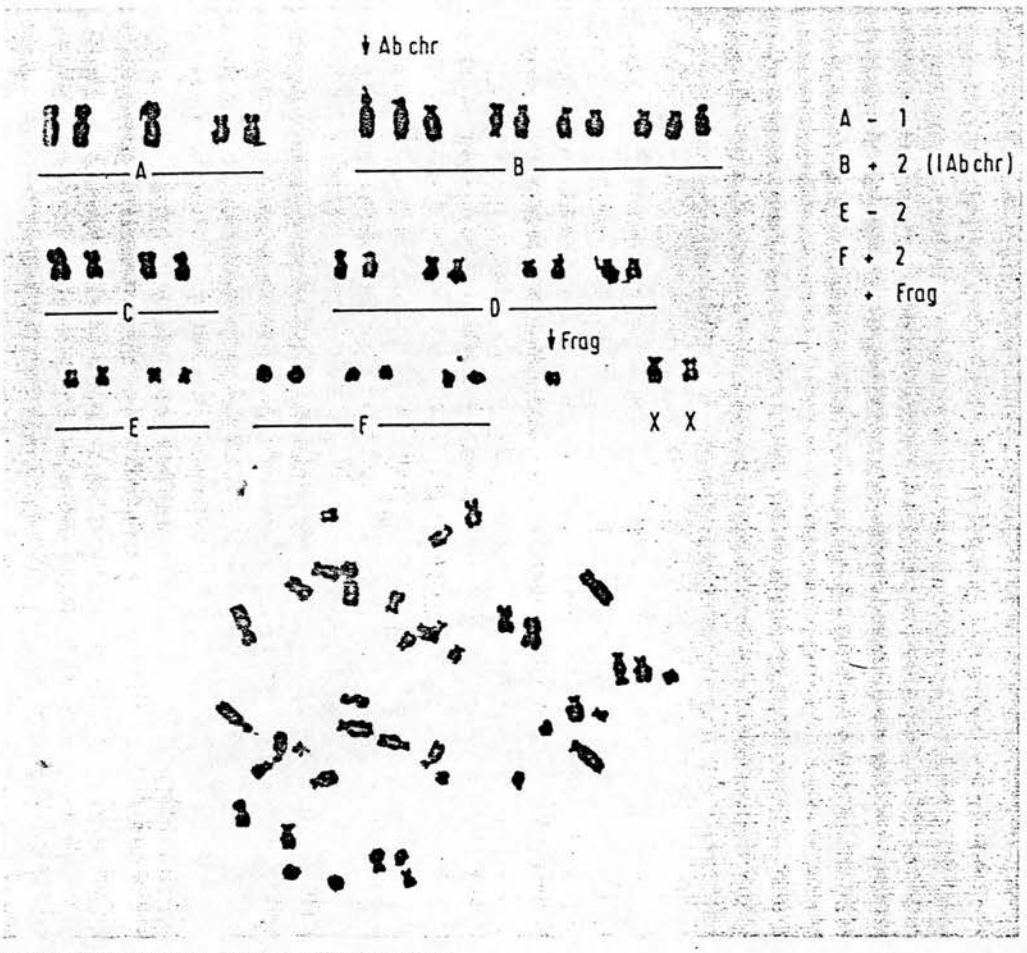


FIG 4: Karyotype of a JM cell showing 39 chromosomes

was 48 hours, and 96 hours in 1 per cent serum. These times remained unaltered throughout 30 passages in vitro. JM cells were also tested for their ability to form colonies in semi-solid agar. Cells from passage 4 were suspended in medium containing 0·25 per cent agarose. Colonies were formed, visible by eye, after 21 days incubation at a cloning efficiency of 4 per cent. Epithelial strain A and the fibroblastic strain did not form colonies, while epithelial strain B formed very small colonies at an efficiency of less than 1 per cent, just visible by eye after 21 days. The JM clones were picked using a micropipette and cultured separately. At the light microscope level and from measuring rate of growth, they did not differ from the parent cells.

The cell surfaces were examined for fibronectin content using an indirect immunofluorescence technique (Norval et al 1984a). JM cells showed positive immunofluorescence as did the two epithelial cell strains, fibronectin being present in about the same quantity as measured by dilution of the antiserum to fibronectin. Sugars on the cell surface were studied using a direct immunofluorescence test with 11 lectins labelled with FITC (Norval et al 1984a). Wherever possible the specificity of the binding was demonstrated by a reduction of immunofluorescence in the presence of the sugar to which the lectin binds. The results are shown in Table 1 where it may be seen that JM cells were generally the same as for the two epithelial cell strains with three exceptions. In two cases, SBA and DBA, both of which have specificity for N-acetyl D-galactosamine, the lectin was bound to JM cells and not to the two epithelial cell strains, while with RCA-I which has specificity for B-linked D-galactose, the converse was true.

If the JM cells were treated with 5-iodo-2′-deoxyuridine followed by dexamethasone, with progesterone or with luteotropic hormone, no retroviruses

NIH3T3 system while they are present in some human carcinomas of the colon, lung and pancreas (Pulciani et al 1982).

The JM cells were tumorigenic in CBA athymic nude mice, the tumours produced being similar in histopathology to the original feline tumour. If such tumours were excised, disaggregated with collagenase/dispase and cultured in vitro, they produced cells with the same morphology and pattern of growth as the original JM cells. It will be interesting to look at hormone receptors in these cells, tumour induction in male nude mice and to study the effects of antioestrogenic compounds like tamoxifen.

As far as the authors know, this is the first tumorigenic feline mammary cell line to be described and, in view of the similarities in the nature and biological behaviour of feline and human breast carcinomas, further study of these cells should yield information of comparative significance.

Acknowledgements

We thank Dr C. Marshall for the NIH3T3 cells and Dr J. Kulski for the C-127 cells. The work was supported by the Wellcome Trust.

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Received for publication July 2, 1984

Association of toxin-producing fungi with disease in broilers

J. ROBB, MSc. *East of Scotland College of Agriculture, Kings Buildings, West Mains Road, Edinburgh,* K. S. KIRKPATRICK, MRCVS, BVMS. *D. B. Marshall (Newbridge) Ltd, Newbridge, Midlothian,* M. NORVAL, PhD, BSc. *Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh*

Veterinary Record (1982) **111**, 389-390

An episode of suboptimal growth, poor feathering and behavioural abnormalities in broilers in Scotland during the winter of 1980-81 is described. This was considered to be associated with mould-contaminated maize and wheat components of the feed, from which fusaria were isolated in persistently high numbers. Four species, *Fusarium culmorum, F tricinctum, F nivale* and *F moniliforme*, were identified. Chloroform extracts of the raw materials and of an artificial medium in which three of the *Fusarium* species were cultured proved toxic to tissue cultures of a human epithelial cell line (HEp II). Specific identification by thin layer chromatography of the mycotoxins deoxynivalenol, zearalenone and diacetoxyscirpenol was achieved in some extracts. In addition, several other areas of the chromatograms were found to be toxic in the HEp II cell system and these may contain toxins for which standards were not available or, alternatively, previously uncharacterised fungal metabolites. It was concluded that the toxins produced by the fusaria were major contributing factors to the disease symptoms shown by the birds.

IN 1960 there was an outbreak of aflatoxicosis (turkey X disease) in poultry in Britain (Butler 1973). Thousands of turkeys died after eating rations containing groundnut meal contaminated by *Aspergillus flavus* which had produced aflatoxin. Since then no mycotoxin-associated disease episode in any animal species has been described, despite these metabolites being present in some types of animal feedstuffs (Roberts and others 1975). However, it has been reported that mycotoxins of the trichothecenes group, which are produced by various *Fusarium* species, may be involved in altered feathering and reduced growth in poultry (Wyatt and others 1973). There is, therefore, the potential for these compounds to affect bird performance, especially where dietary parameters are closely controlled.

An episode of toxin-related disease seen in broilers in Scotland during the winter of 1980-81 is reported here. The subsequent investigation indicated that the presence of mycotoxins in some components of the feedstuffs may have been the major contributing factor.

Clinical symptoms

In general, the flocks were kept in groups of 18,000 in houses of approximately 10,000 square feet floor area. All were warm air brooded in part of the house only (3500 square feet) and were fed standard broiler diets supplied from one mill.

The time of onset of clinical signs varied but on several occasions was in five-day-old chicks. Affected chicks showed a high level of excitability, excessive activity, high pitched chirping and wall-pecking. There was a tendency for birds to stand in groups and some individuals within groups stood passively allowing their toes to be pecked. Examination revealed that a number of these birds were suffering from varying degrees of vasoconstrictive damage to one or more toes, with well defined dry necrosis being evident in most cases. Ultimately, sloughing of the affected toes took place.

There was a general lack of interest in food within these groups and selective feeding was evident. Small birds and toe damaged birds were the least inclined to eat and therefore the size distribution of birds within the house widened. Mortality in the first and second weeks was 1 per cent and 0·5 per cent respectively above normal levels, and post mortem examinations revealed dehydrated birds with slightly enlarged livers and grossly distended gall bladders. A variety of colours was present in the livers but no significant or characteristic histopathological feature was present.

During the second to fourth week of life, uneven growth was apparent within the affected flocks and up to 10 per cent of small poorly feathered birds were detected. These birds were well muscled with pendulous abdomens; feather growth was slow and feather quality poor. On culling and post mortem examination, the only regular finding was an obvious thickening of the cartilage growth plates, particularly in the long bones of the legs. The birds appeared rickety but this was not confirmed histologically.

Individual birds with nervous involvement associated with head tremors were detected in several flocks and, on two occasions, about 10 per cent of two, four-week-old flocks suffered acute incoordination and collapse with loss of the righting reflex. The episode occurred four to six hours after the introduction of a new batch of feed. Gradual recovery of affected birds took place over 12 hours with very limited mortality being recorded.

Periods of excessive thirst, soft dropping and an increase in the level of faecal fat excretion were seen in all affected groups. Between five-and-a-half and eight weeks of age, some degree of compensatory growth occurred in most flocks. However, the variability in bird size with unacceptably high food consumption continued to be apparent at the end of growout.

In a comparative feed trial using known toxic feed and a normal research diet of similar nutrient specification, the weight achievement of test chicks to 14 days of age was 56 per cent that of the control group.

Microbiological tests

No pathogenic bacteria were isolated from the necrotic toe lesions, from the small culled chickens or from the feed. In addition, the disease could not be reproduced by taking whole intestines and contents from affected birds and inoculating a homogenate of these into the crops of one-day-old broiler chicks using the method described by Kouwenhoven and others (1978). Nor did the disease appear to have the characteristics of horizontal spread caused by an infectious agent. Its geographical distribution and speed of onset seemed to indicate that it was feed associated.

Feed from affected houses was examined for the presence of fungi. Samples were diluted with Ringer's solution and surface plated on potato dextrose agar, malt extract agar and half strength tryptone soya agar supplemented with casein hydrolysate. Incubation temperatures were 25°C, 37°C and 55°C respectively.

Fusarium species, between 10^5 and 10^6/g, were isolated; the normal numbers found in this part of Britain are about 10^3 to 10^4/g. Four species were identified: *Fusarium culmorum, F tricinctum, F nivale* and *F moniliforme*, with the latter predominating. It is known that many *Fusarium* species have the ability to produce various toxic metabolites and therefore the

presence of an abnormally large population of these organisms indicated the potential for the production of significant toxins.

Mycotoxin testing

Feedstuffs

Feedstuffs and their individual components were examined for the presence of toxins and for toxigenic capacity. In all the analyses, 25 g samples were extracted according to the method of Patterson and Roberts (1979) and the concentrated chloroform and aqueous extracts were analysed by thin layer chromatography using Patterson and Roberts' standards plus deoxynivalenol. The trichothecenes diacetoxyscirpenol and deoxynivalenol were positively identified in the maize and wheat components of the feedstuff. This was later confirmed by gas-liquid chromatography.

In addition, biological assay of the extracts using a human epithelial cell line (HEp II) as an indicator system was performed, in a similar manner to that described by Saito and others (1971). The cells were grown on coverslips in 1 ml Earles-based Eagle's complete medium supplemented with 5 per cent new born calf serum, 200 iu/ml penicillin and 200 μg/ml streptomycin until semiconfluent. Thereafter small aliquots (0.5, 1 and 2 μl) of the chloroform and aqueous extracts were added. After 48 hours incubation at 37°C the coverslips were removed from the medium, the cells fixed in ethanol and stained by Giemsa, and examined microscopically to assess the cytotoxic effect.

Of the 18 standard mycotoxins available, it was found that 10 were toxic in such a test, namely ochratoxin A, diacetoxyscirpenol, T-2 toxin, T-2 triol, PR-toxin, T-2 tetraol, roridin, deoxynivalenol, verrucarin A and HT-2 toxin. A clear cytotoxic effect on the HEp II cells was observed with the chloroform extracts of the feedstuffs (Figs 1 and 2). No such effect was seen in cells incubated with the aqueous extracts or with chloroform extracts from batches of feedstuffs not producing adverse symptoms in birds.

Fusaria

The four *Fusarium* species were cultured in 50 ml liquid potato dextrose medium for 10 days at 25°C, followed by two weeks at 10°C. This procedure was considered to maximise toxin production from several *Fusarium* species (J. Robb and M. Marsh, unpublished data). The medium was then extracted as described and on bioassay it was found that the chloroform extracts of *F culmorum*, *F moniliforme* (Fig 3) and *F tricinctum* contained compounds toxic to the HEp II cells. Zearalenone was identified in the *F culmorum* extract and diacetoxyscirpenol in the *F moniliforme* extract by thin layer chromatography and confirmed by gas-liquid chromatography.

In addition, several spots were seen on the thin layer chromatograms, plus the track from the *F tricinctum* extract,

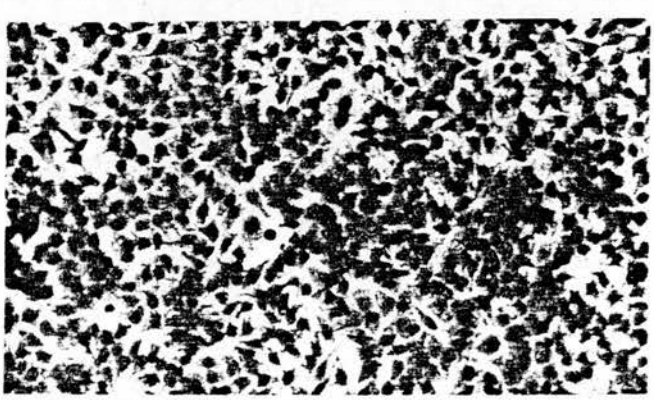

FIG 1: HEp II cells (Giemsa × 117)

FIG 2: HEp II cells after incubation for 48 hours with 0.5 μl chloroform extract of feedstuff (Giemsa × 117)

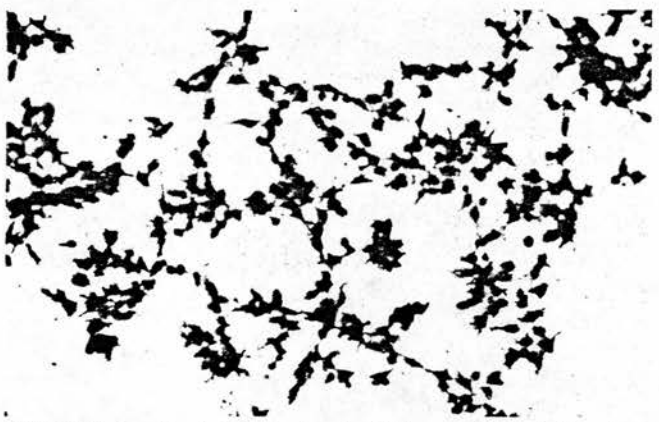

FIG 3: HEp II cells after incubation for 48 hours with 1 μl chloroform extract of *F moniliforme* (Giemsa × 117)

which did not correspond to the available standards. When eluted, some of these areas were toxic in the HEp II test; these may represent additional (as yet) unidentified mycotoxins.

Growing the individual *Fusarium* species on sterilised grain at 25°C for 10 days, then at 10°C for two weeks, produced extracts with similar characteristics to those observed in the extracts of the culture media described above.

Conclusions

The feedstuffs associated with disease symptoms in broilers contained higher than normal numbers of *Fusarium* species, and several of these species were capable of producing toxic metabolites when cultured on a medium or on sterilised grain. The commercial diets contained similar toxic compounds. It is therefore concluded that their presence in the feed was an important contributory factor in the disease episode described. We urge consideration of mycotoxins as factors in animal diseases, especially where diet and weight gain are tightly controlled and where feed is mixed and given continuously from bulk supplies.

Acknowledgements.—We acknowledge the expert advice given by Mr C. Randall and the valuable assistance of Miss Margo Marsh, Mrs Ann Arthur and Miss Barbara Robinson.

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0099-2240/83/100948-03$02.00/0

Vol. 46, No. 4

Comparison of Cytotoxicity and Thin-Layer Chromatography Methods for Detection of Mycotoxins

JEAN ROBB[1]* AND MARY NORVAL[2]

Department of Microbiology, East of Scotland College of Agriculture,[1] and Department of Bacteriology, University of Edinburgh Medical School,[2] Edinburgh, Scotland

Received 11 April 1983/Accepted 14 July 1983

Thirty-three standard mycotoxins were assayed by thin-layer chromatography and by cytotoxicity in HEp-2 and Chang cells. Various levels of detection were found. The cytotoxicity test was significantly more sensitive than thin-layer chromatography for the trichothecenes and should be useful for screening extracts from animal feedstuffs for the presence of unknown mycotoxins.

It has become increasingly obvious in recent years that outbreaks of disease may occur in farm animals in Britain owing to the ingestion of various mycotoxins in feedstuffs. In 1960, an episode of aflatoxicosis (Turkey X disease) occurred when many thousands of turkeys died after eating rations containing groundnut meal contaminated by *Aspergillus flavus* which had produced aflatoxin (reviewed in reference 1). More recently, toxins elaborated by various species of *Fusarium* in the maize and wheat components of the feed were implicated in an outbreak of suboptimal growth in broiler chickens (8). It is thus of importance to be able to screen various animal feedstuffs for the presence of mycotoxins and to assay for these compounds in outbreaks of disease of unknown etiology. A multimycotoxin-screening procedure has been described by Patterson and Roberts (7) and involves extraction of the feedstuff with organic solvents followed by thin-layer chromatography (TLC) and identification by comparison with mycotoxin standards. This screen is therefore confined to the identification of known toxins and, in addition, is relatively insensitive for the detection of some compounds.

Various biological methods have been described for the assay of mycotoxins, ranging from whole animals such as brine shrimp (3) and chicken embryos (5) to in vitro tissue culture systems such as human embryo fibroblasts (2), rabbit reticulocytes (4), and HeLa cells (9). The range of mycotoxins which may be detected by a given assay has been rarely characterized; also, the relative sensitivities of the various systems have not been compared. Therefore, it was thought useful to develop a standard biological assay which could be carried out routinely with ease and to compare the sensitivity and range of mycotoxins detected with a conventional assay employing TLC.

There were 33 standard preparations of mycotoxins available which had been tested for purity and identity by nuclear magnetic resonance spectroscopy. The sources of these were aflatoxin G_1, cytochalasin B and D, scopoletin, and T-2 toxin from Sigma Chemical Co.; aflatoxins B_1, B_2, G_2, and M_1, citrinin, diacetoxyscirpenol, HT-2 toxin, luteoskyrin, 5-methoxysterigmatocystin, *O*-methyl sterigmatocystin, ochratoxin A, patulin, penicillic acid, PR toxin, rubratoxin B, sterigmatocystin, T-2 tetraol, and zearalenone from Makor Chemicals, Israel; roridin, T-2 triol, verrucarin A, and verrucarol from Cambridge Research Biochemicals Ltd.; 4-acetoxy-3,15-scirpendiol, 15-acetoxy-3,4-scirpendiol, scirpentriol, triacetoxyscirpene isolated from *Fusarium sulphurium* (10), and moniliformin isolated from *Fusarium monoliforme*, all kindly donated by P. S. Steyn; and deoxynivalenol kindly donated by M. Shepherd. Each was dissolved in acetone at 1 mg/ml, and 10-fold serial dilutions were made in acetone. Controls contained acetone alone.

For the TLC, plates (Merck art. 5554 DC-Alufolien Kieselgel 60F 254 and art. 5553 DC-Alufolien Kieselgel 60) were spotted with 5μl samples of each standard or dilution of standard, run in toluene-ethyl acetate-formic acid (60:30:10), and sprayed with 50% aqueous H_2SO_4 when necessary, before viewing in a UV cabinet (shortwave, 254 nm; longwave, 366 nm). R_f values are noted in Table 1. The lowest dilution at which spots could be detected by eye was noted.

For the biological assays, two cell lines were used, HEp-2 cells and Chang cells, both human epithelial type, the latter of liver origin. These were maintained on Earles-based Eagles medium supplemented with 5% newborn calf serum, 200 IU of penicillin per ml, and 200 μg of streptomycin per ml, and subcultured by tryp-

TABLE 1. Lowest levels at which mycotoxins were detected in various tests

Mycotoxin	Nanograms of mycotoxin			R_f values
	Cytotoxicity		TLC	
	HEp-2	Chang		
4-Acetoxy-3,15-scirpendiol	100	10	50	0.14
15-Acetoxy-3,4-scirpendiol	100	10	500	0.09
Aflatoxin B_1	100	100	0.5	0.17
Aflatoxin B_2	100	100	0.5	0.13
Aflatoxin G_1	ND[a]	ND	0.5	0.14
Aflatoxin G_2	1,000	100	0.5	0.11
Aflatoxin M_1	10	10	0.5	0.0
Citrinin	10	100	0.05	0.42
Cytochalasin B	ND	ND	500	0.3
Cytochalasin D	10	10	50	0.17
Deoxynivalenol	100	100	500	0.0
Diacetoxyscirpenol	1	10	500	0.23
HT-2 toxin	10	1	500	0.18
Luteoskyrin	ND	ND	500	0.38
5-Methoxysterigmatocystin	Not done	ND	5,000	0.66
O-Methyl sterigmatocystin	100	10	5,000	0.32
Moniliformin	ND	ND	5,000	0.0
Ochratoxin A	ND	ND	0.05	0.45
Patulin	100	100	500	0.32
Penicillic acid	1,000	1	50	0.35
PR toxin	1	100	50	0.44
Roridin A	0.0001	0.1	500	0.26
Rubratoxin B	ND	ND	ND	0.22
Scirpentriol	100	10	50	0.04
Scopoletin	ND	ND	0.5	0.27
Sterigmatocystin	100	10	5,000	0.56
T-2 tetraol	10	100	500	0.04
T-2 toxin	10	0.01	500	0.26
Triacetoxyscirpene	10	10	500	0.38
T-2 triol	100	100	500	0.1
Verrucarin A	0.1	0.1	5,000	0.35
Verrucarol	1,000	1,000	50	0.15
Zearalenone	100	100	50	0.55

[a] ND, Not detected at 1,000 ng.

sinization when confluent. In the test, they were cultured on cover slips in 1 ml of medium until semiconfluent. One microliter of each standard or dilution of standard was added, and after 48 h of incubation at 37°C, the cover slips were removed, the cells were fixed in ethanol, stained with Giemsa stain, and examined microscopically to assess the cytotoxic effect, if any. The lowest dilution at which a cytocidal effect was apparent was noted, i.e., marked inhibition of growth, cell rounding, and detachment from the surface. Each observation was made in triplicate, and cytotoxic levels were found to vary by a maximum of 1 dilution. The results are given in Table 1.

It may be seen that there was great variation in the level at which the standard mycotoxins were detected in the assay systems used, from no effect at 1,000 ng to visible spots at 0.05 ng in the TLC for citrinin and ochratoxin A, and cytotoxicity at 0.0001 ng for roridin A in HEp-2 cells. Although the TLC method detected almost all of the compounds used, it was not as sensitive as the cytotoxic assay for at least 13 of the 33 mycotoxins tested. This difference in sensitivity was most striking for the trichothecenes, for example, T-2 toxin and verrucarin. The cytotoxic test did not detect eight compounds with either Chang or HEp-2 cells, and generally there was some difference in the level of sensitivity between the two lines. Lompe and Milczewski (6) described the effect of 16 mycotoxins on three human and two porcine cell lines and found various levels of sensitivity, suggesting the use of more than one line of different origin in screening. The level of sensitivity of HEp-2 cells to roridin A was higher than expected but was reproducible in three separate series of dilutions and cytotoxic tests.

Thus, when screening for mycotoxins in such materials as animal feedstuffs, it would be advantageous to use a cytotoxic assay system in

addition to TLC. Apart from the increased sensitivity for the trichothecene group, the cytotoxic method has a good chance of picking up unknown toxins which may be present in samples of feedstuff associated with outbreaks of disease and for which there are no standards available.

We thank T. J. Simpson for supplying some of the mycotoxins used in this work and for helpful discussions.

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VETERINARY AND MEDICAL IMPLICATIONS OF MYCOTOXINS

Trichothecenes and Other Mycotoxins
Edited by J. Lacey

CHAPTER 35

The use of a cytotoxicity test as a screening test for mycotoxins

Jean Robb and Mary Norval

INTRODUCTION

The ability of fungi to produce mycotoxins in forages and stored feedingstuffs is well known. As a result of this potential hazard, or because of specific disease problems or sub-optimal performance in poultry and animals, animal feedingstuffs are routinely screened for mycotoxins. Usually solvent extracts of feedingstuffs are subjected to a clean-up stage and then analysed by thin-layer, gas–liquid or high-pressure liquid chromatography. In many instances multimycotoxin methods (Gorst-Allman *et al.*, 1979) are used, making it possible to extract several mycotoxins of different chemical structures in the one procedure. These methods are often relatively insensitive, particularly for trichothecene toxins, and are only able to detect compounds of known chemical composition providing the necessary standards are available.

It is possible therefore that animals may eat feeds containing mycotoxins which routine laboratory methods cannot detect. Such a situation occurred in Scotland during the winter of 1980–81 when sub-optimal growth, poor feathering and behavioural abnormalities were seen in broilers (Robb *et al.*, 1982). Examination of poultry meal extracts by thin-layer chromatography (Patterson and Roberts, 1979) failed to demonstrate mycotoxins but bioassay with a tissue culture of HEpII cells enabled identification of the toxic component of the feed. Since then the sensitivities of the cytotoxicity and thin-layer chromatography methods have been compared using toxin standards and extracts from feedingstuffs. This chapter describes the results of these experiments.

METHODS

Thirty-three standard preparations of mycotoxins were used; they were aflatoxin G_1, cytochalasin B and D, scopoletin (Sigma), aflatoxin B_1, B_2, G_2

and M_1, citrinin, diacetoxyscirpenol, HT-2 toxin, luteoskyrin, 5-methoxysterigmatocystin, O-methyl sterigmatocystin, ochratoxin A, patulin, penicillic acid, PR-toxin, rubratoxin B, sterigmatocystin, T-2 tetraol, T-2 toxin and zearalenone (Makor Chemicals, Israel), roridin, T-2 triol, verrucarin A and verrucarol (Cambridge Research Biochemicals Ltd), 4-acetoxy-3,15-scirpendiol, 15-acetoxy-3,4-scirpendiol, scirpentriol, triacetoxyscirpene and moniliformin (all kindly donated by Dr P. S. Steyn) and deoxynivalenol (kindly donated by Dr M. Shepherd). Each was dissolved in acetone at 1 mg/ml and 10-fold serial dilutions made in acetone.

Animal feedingstuffs were extracted with acetonitrile, iso-octane and chloroform and then examined by thin-layer chromatography (Patterson and Roberts, 1979; Figure 1).

For TLC, plates (Merck Art 5554 DC-Alufolien Kieselgel 60F 254 and Art 5553 DC-Alufolien Kieselogel 60) were spotted with 5 μl samples of each concentration, run in toluene : ethyl acetate : formic acid 60 : 30: 10, and sprayed with 50% aqueous H_2SO_4 where necessary, before viewing in a UV cabinet (short-wave 254 nm, long-wave 366 nm). The lowest dilution at which spots could be detected visually was noted.

For biological assay, HEpII cells were used. The cells were maintained on coverslips in 1 ml Earles-based Eagles medium supplemented with 5% newborn calf serum, 200 i.u./ml penicillin and 20 μg/ml streptomycin, and subcultured by trypsinization when confluent. In the test, they were cultured on coverslips in 1 ml medium until semi-confluent. One microlitre (1 μl) of each dilution of the standard or 1 μl of feedingstuff extract was added and, after 48 hours incubation at 37 °C, the coverslips were removed, the cells fixed in ethanol, stained by Giemsa's and examined microscopically to assess cytotoxicity. The lowest dilution at which a cytocidal effect, i.e. marked inhibition of growth, cell-rounding and detachment from the surface, was apparent was noted. Observations were made in triplicate and cytotoxic levels were found to vary by a maximum of 1 dilution.

RESULTS

Table 1 shows that there was much variation in the level at which the standard mycotoxins were detected in the assay systems employed, from undetectable at 1000 ng to visible spots at 0.05 mg in TLC of citrinin and ochratoxin A respectively or cytotoxicity to HEpII cells at only 0.0001 ng of roridin A. While the TLC method detected most compounds, it was less sensitive than the cytotoxic assay for at least 13 of the 33 tested. This difference in sensitivity was most striking for the trichothecenes, e.g. T-2 toxin and verrucarin A.

The results in Table 2 show that, apart from the turkey feeds and the tapioca, no individual feedingstuff was consistently toxic to HEpII cells.

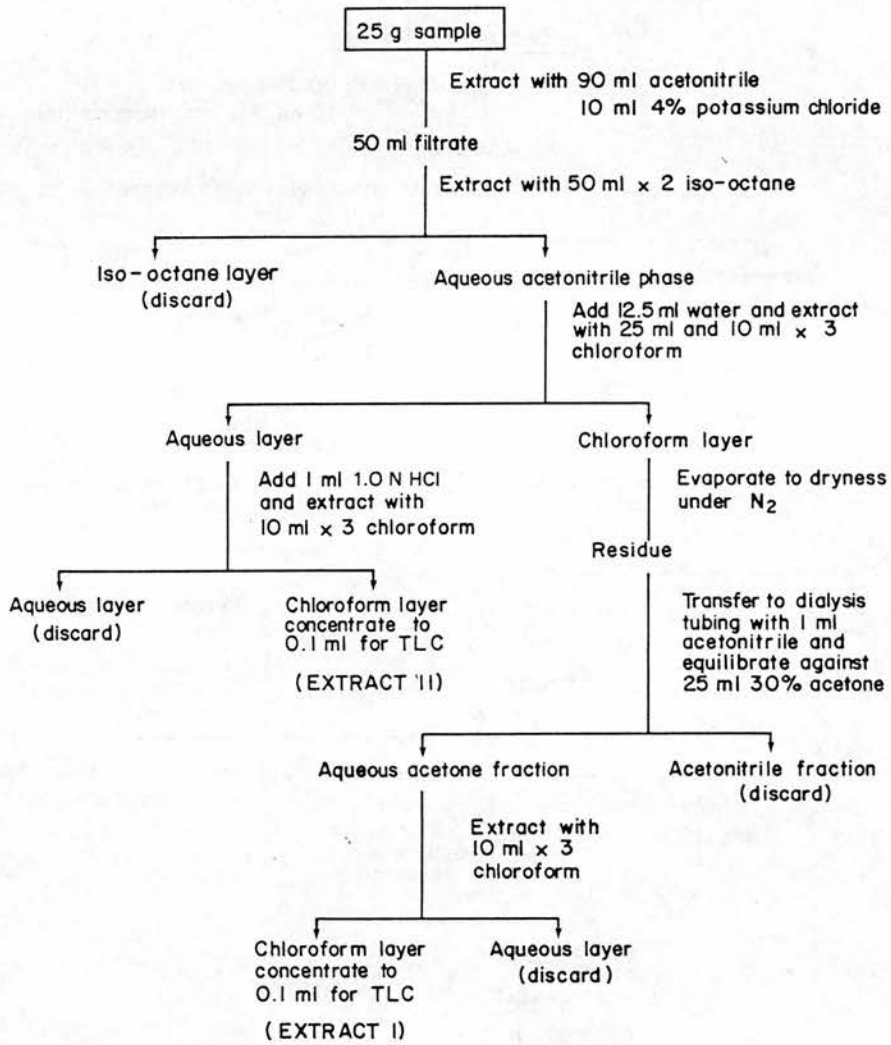

Figure 1 Extraction and clean-up procedure for the detection of mycotoxins in animal feeds.

However, few of these were tested, and these were directly related to a disease problem so that the results may not be representative. A supplement used in the poultry feeds was consistently toxic, but its effects in the final feed were diluted.

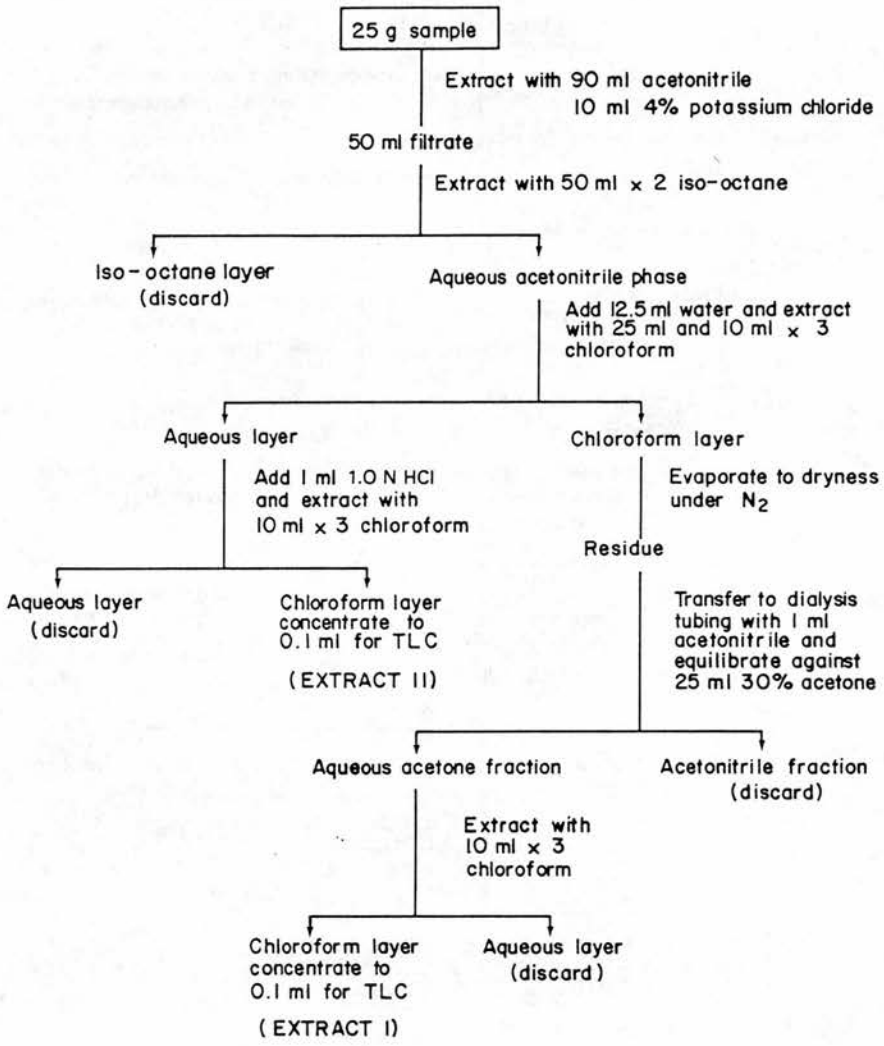

Figure 1 Extraction and clean-up procedure for the detection of mycotoxins in animal feeds.

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Table 2 Cytotoxicity

Feed type	Cytotoxicity extract I		Cytotoxicity extract II	
	+ve	−ve	+ve	−ve
Barley	1	5	0	6
Wheat	7	14	1	20
Starter pellets	33	20	7	46
Maize	8	18	3	23
Starter crumbs	22	7	11	18
Broiler finisher	9	1	3	7
Turkey feed	2	0	2	0
Soya	6	5	3	8
Fat	1	2	1	2
Soffe	0	1	0	1
Supplement	3	0	1	2
Tapioca	1	0	0	1
Silage	6	18	0	22
Cattle cake	2	17	0	19
Grass	0	4	0	4
Sow mix	2	12	0	14
Potatoes	0	4	0	4
Draff	6	15	1	20
Oats	1	10	0	11
Straw	0	6	0	6

feedingstuff may be responsible for the toxicity. Untreated cereals or forages are less likely to contain interfering compounds. Apart from demonstrating sensitivity to the trichothecenes, the test will also indicate the presence of other mycotoxins, known and unknown, which are not included in the routine standards used for TLC or GLC analysis.

Lompe and Milezewski (1979) described the effect of 16 mycotoxins on three human and two porcine cell lines and found varying levels of sensitivity, suggesting that several cell lines of different origin might be used in screening. If extracts were passed through a clean-up cartridge such as Sep-Pak and aliquots tested against various cell lines, feedingstuffs which gave positive results could be re-extracted and analysed by the most sensitive chemical method available for the types of toxic compounds indicated by the cytotoxicity tests.

ACKNOWLEDGEMENTS

We wish to thank Dr T. Simpson for supplying some of the mycotoxins used in this work and for helpful advice. We are also grateful to Dr Brian Flannigan for helpful criticism.

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University of Edinburgh Medical School, Edinburgh, U.K.

335. Interactions between herpes simplex type I and murine bone marrow macrophages

SARAH HOWIE, MARY NORVAL, and J. MAINGAY

The association of a cloned clinical isolate of herpes simplex type I (HSV-I) with bone marrow derived macrophages from C3HfBu/Kam mice has been investigated. The bone marrow cells were prepared from the femurs of 6–8 week old mice and cultured in L-cell conditioned medium. On days 0, 2, 4, 6 and 8 they were infected with HSV-I at a multiplicity of 1, and the outcome was followed in various ways. Infectious centre assays were performed, and infectious virus present in the culture supernatant and cell-associated at 1 day and 3 days

post-infection was measured by a plaque assay. The number of cells expressing viral antigens was estimated by indirect immunofluorescence at the same times after infection. In addition the uptake of ^{3}H-thymidine into the infected cells over a 24-hour period after infection was compared with uninfected controls. Finally the Fc receptor avidity of adherent cells, and the expression of cell surface class II MHC antigens were measured at 1 and 3 days post infection. In uninfected cultures the number of adherent cells with high avidity Fc receptors increased from 15 % on day 1 to 70 % on day 3, and greater than 90 % after further incubation. In infected cultures 3 days post-infection, greater than 90 % of the surviving cells had high avidity Fc receptors, although there were only 10 % of the cells remaining compared to uninfected controls. This suggests that the cells which can survive such an infection are a subpopulation of mature macrophages. Throughout the culture period, a small percentage of cells (0.2 %) permissive for HSV replication and expressing viral antigens were present and these increased 6–8 days after the culture had been initiated. DNA synthesis was inhibited by 50 % in infected cultures suggesting that, even in non-permissive cells, host macro-molecular synthesis may be inhibited by the virus. Similar experiments using bone marrow derived macrophages from neonatal mice are presently being undertaken. These macrophages represent one class of antigen presenting cell, and a study of their interactions with HSV may clarify the types of immune responses generated by different antigen presenting cells during infection.

Archives of Virology 87, 229—239 (1986)

Archives of Virology

Interactions Between Herpes Simplex Virus and Murine Bone Marrow Macrophages

By

Sarah Howie, Mary Norval, J. Maingay, and W. H. McBride*

Department of Bacteriology, University of Edinburgh, Medical School, Edinburgh, United Kingdom

With 5 Figures

Accepted May 13, 1985

Summary

Macrophages from murine bone marrow (strain C3Hf Bu/Kam) were cultured *in vitro* in L-cell conditioned medium. After 0, 2, 4, 6, 8 and 10 days, they were infected with a clinical strain of herpes simplex virus type 1 and the outcome followed morphologically, by phagocytic index, infectious virus yields, immunofluorescence, expression of Fc receptors and major histocompatibility complex (MHC) Class II antigens. At a multiplicity of infection of 1—5, little morphological difference was apparent between infected and uninfected cultures at early stages *in vitro* but marked changes occurred later with reduction in cell numbers in the infected cultures. Indirect immunofluorescence failed to detect cells expressing early viral antigens, and yields of infectious virus indicated that permissive infection was not taking place. While phagocytic index and Fc receptor expression did not change 24 hours post-infection, MHC Class II antigen expression was increased. Thus, although the bone marrow macrophages seem predominantly resistant to infection with HSV-1, they may be modified by the presence of the virus. Since macrophages may act as antigen presenting cells for the immune system, this type of mechanism may be important in the generation of local immune responses.

Introduction

Macrophages play a crucial role in the generation of both humoral and cell-mediated immune responses acting as antigen presenting cells, and are

* Present address: Department of Radiation Oncology, UCLA Centre for Health Sciences, Los Angeles.

recognised to be of primary importance in resistance to viral infections. Several studies have indicated that they are necessary for resistance to herpes simplex virus (HSV) infections in mice; if macrophages are depleted or immature, then resistance to HSV is dimished and, conversely, agents which stimulate macrophages increase resistance (3, 5, 19). Most work on the function of macrophages in viral infection has involved peritoneal cells and this subject has been reviewed recently by MORAHAN (12).

It was reported in 1971 that peritoneal macrophages can be abortively infected with HSV type 1 (HSV-1) with a possible block at the stage of virion assembly (17). While peritoneal macrophages are generally considered non-permissive for HSV replication unless stimulated *in vivo* by agents such as thioglycollate, they can be made permissive by pre-culture *in vitro* for 3 to 4 days before infection (9). Recently, several strains of HSV-1 were found to replicate to different extents in peritoneal macrophages after they had been aged *in vitro* or thioglycollate stimulated. This ability could not be correlated with pathogenicity in mice (2). Different results were reported by BRUCHER *et al.* (1) who showed an association between the permissiveness of peritoneal macrophages to HSV-1 and *in vivo* susceptibility to infection. Macrophages from resistant strains of mice produced more interferon post-infection than macrophages from more sensitive strains, suggesting a possible mechanism of selective resistance.

Macrophages are morphologically and functionally diverse. They are thought to originate from precursor monoblasts and promonocytes in the bone marrow. If bone marrow cells are cultured *in vitro*, in the presence of L-cell conditioned medium which contains "macrophage colony stimulating factor", they are stimulated to differentiate and divide, and have been shown by morphology, esterase staining and phagocytosis to provide a culture of virtually pure macrophages (6). Thus these cells provide a convenient model system for studying the association of HSV with macrophages of different maturation states. This may help to clarify the types of immune responses generated by antigen presenting cells during the course of a viral infection.

Bone marrow macrophages were obtained from mice and infected with HSV-1 after various periods in culture. The outcome of the infection was followed morphologically, by phagocytic index, infectious virus yields, immunofluorescence, and by expression of Fc receptors and major histocompatibility complex (MHC) Class II antigens.

Materials and Methods

Bone Marrow Cultures

Femurs from 6—8 week old female mice (C3Hf Bu/Kam strain, bred and maintained in the Departmental Animal House) were collected and bone marrow cells prepared by cutting off the ends and flushing aseptically with medium, the yield

being about 10^7 cells per femur. The cells were washed and cultured at various densities as described below. The medium used throughout was RPMI 1640 (Imperial Laboratories) supplemented with 10 to 30 per cent L-cell conditioned medium (depending on the batch used), 5 per cent foetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, 200 μg/ml streptomycin and 100 μg/ml gentamicin. The conditioned medium was prepared from pooled 24-hour supernatants from confluent L-cell cultures which were filter sterilized, titrated to find the optimal concentration for growth of bone marrow macrophages and stored at −70° C until use.

During the culture period, the percentage of adherent macrophages present was determined by a non-specific esterase strain (18) and by phagocytosis of sensitized calf red blood cells.

Virus and Viral Infections

HSV-1 was isolated from a clinical case and was plaque-purified in Vero cells. These cells were cultured in Eagle's minimal essential medium (Northumbria Biologicals Ltd.) supplemented with 2 to 5 per cent new born calf serum, 2 mM L-glutamine and antibiotics as above. Plaque assays for infectious virus were performed using Vero cells in microtitre plates with 0.25 per cent Seaplaque agarose (FMC Corporation) in the overlay. After incubation for 3 days, the cells were fixed in formyl saline and stained with Giemsa before counting the plaques using a low power microscope. The same batch of virus stock was used throughout, and it contained 10^{10} plaque forming units (PFU) per ml.

On day 0, for all the tests, bone marrow cells were infected as monolayers with the same amount of virus. HSV-1 was allowed to adsorb for 1 hour at 37° C before washing the cell sheets 3 times and putting on fresh medium. All incubations were carried out at 37° C in an atmosphere of air with 5 per cent CO_2 in a humidified incubator.

Virus Yields

Bone marrow cells were seeded at a density of 10^6 in 1 ml volumes in 24-well Costar plates. At 1 and 24 hours post-infection, the cells were scraped off duplicate wells, using small rubber policemen, into the culture supernatant. All samples were stored at −70° C, then ultra-sonicated for 1 minute before being assayed for PFU on Vero cells in microtitre plates, in quadruplicate. The mean of the number of plaques at suitable dilutions was taken.

In addition, on every second day, cells were scraped off 3 wells and counted to give an average number present per well. Cell counts were also performed 24 and 72 hours post-infection.

Immunofluorescence

The bone marrow cells were seeded at a density of 2×10^6 in 1 ml volumes in tubes containing coverslips. The coverslips were fixed at 0, 1, 4, and 24 hours post-infection in 2 per cent paraformaldehyde in 0.28 M Na cacodylate buffer, pH 6.9, for 10 minutes at room temperature. They were treated with 0.05 per cent Triton X-100 in 0.01 M phosphate buffered saline (PBS), pH 7.2, for 5 minutes at room temperature, then washed in the same buffer.

Two antibody preparations were employed in indirect immunofluorescence. The first was a mouse monoclonal antibody T 181 (used undiluted), directed against an early viral nuclear antigen and kindly donated by CHAN (7). It was followed by a rabbit anti-mouse antiserum used at a dilution of 1/50 (4), then a sheep anti-rabbit FITC conjugate at 1/20 (Wellcome). The second antibody preparation was an antiserum raised in a New Zealand rabbit by 3 subcutaneous injections of 2×10^6 RK_{13} cells harvested 4 hours after infection with HSV-1 at a multiplicity of 5. The first

injection was in Freund's complete adjuvant and was followed at fortnightly intervals by injections in Freund's incomplete adjuvant. The rabbit was bled 2 weeks later. The serum was used at a dilution of 1/20, followed by sheep anti-rabbit FITC conjugate as before.

Fc Receptor Assays

Bone marrow cells were seeded at a density of 3×10^5 in 1 ml volumes in 24-well Costar plates. Fc receptor expression was measured 24 hours post-infection as described previously (10). Briefly 5 per cent (v/v) calf red blood cells (CRBC) in PBS were mixed with an equal volume of a 1/32 dilution of hyperimmune rabbit anti-CRBC serum, incubated at 37° C for 1 hour and washed extensively with PBS. Cultures of bone marrow cells were washed and overlaid with 0.3 ml 2.5 per cent (v/v) suspensions of the sensitized red cells and incubated for 1 hour at room temperature. The wells were washed very gently four times with PBS to remove non-adherent erythrocytes. The percentage of bone marrow cells binding 3 or more erythrocytes at the cell surface was determined using an inverted microscope and counting at least 200 cells from each of 4 wells for each time point.

MHC Class II Antigens

Bone marrow cells were seeded at a density of 2×10^6 in 1 ml volumes in tubes containing coverslips. The coverslips were fixed in 2 per cent paraformaldehyde in 0.28 M Na cacodylate buffer, pH 6.9, for 10 minutes, 24 hours post-infection with HSV. The immunoperoxidase method of RITCHIE *et al.* (14) was used with a mouse anti-class II ($I-a^k$) monoclonal antibody [TIB 93 from the American Type Culture Collection (13)]. At least 500 cells were examined on each coverslip and the number showing positive immunoperoxidase staining was noted.

Results

Morphological Characteristics

Initially the bone marrow cultures consisted of a mixed population of adherent and non-adherent cells (Fig. 1a). During *in vitro* culture, macrophage-like cells began to grow out and by the fourth day micro-colonies were beginning to form which are illustrated in Fig. 1b. The cell numbers decreased from an initial 10^6 per well to 2×10^5 during the first 4 days but thereafter the number remained static. Using a non-specific esterase strain, 43 per cent adherent cells showed staining characteristic of macrophages after 3 days *in vitro*, and this number rose to over 99 per cent by 6 days. Using sensitized CRBC, 10 per cent of the bone marrow cells were phagocytic after 1 day *in vitro*. This increased to 36 per cent after 3 days, and 84 per cent after 7 days (see below and Fig. 2). Thus by these criteria, the bone marrow cells represent a culture of relatively pure macrophages which undergo maturation and proliferation *in vitro* in the presence of L-cell conditioned medium.

After 0, 2, 4, 6, 8 and 10 days *in vitro*, the cultures were infected with HSV-1. Cells from the earlier times (0 and 2 days) showed little morphological change from uninfected controls, but cells from 4 days onwards showed

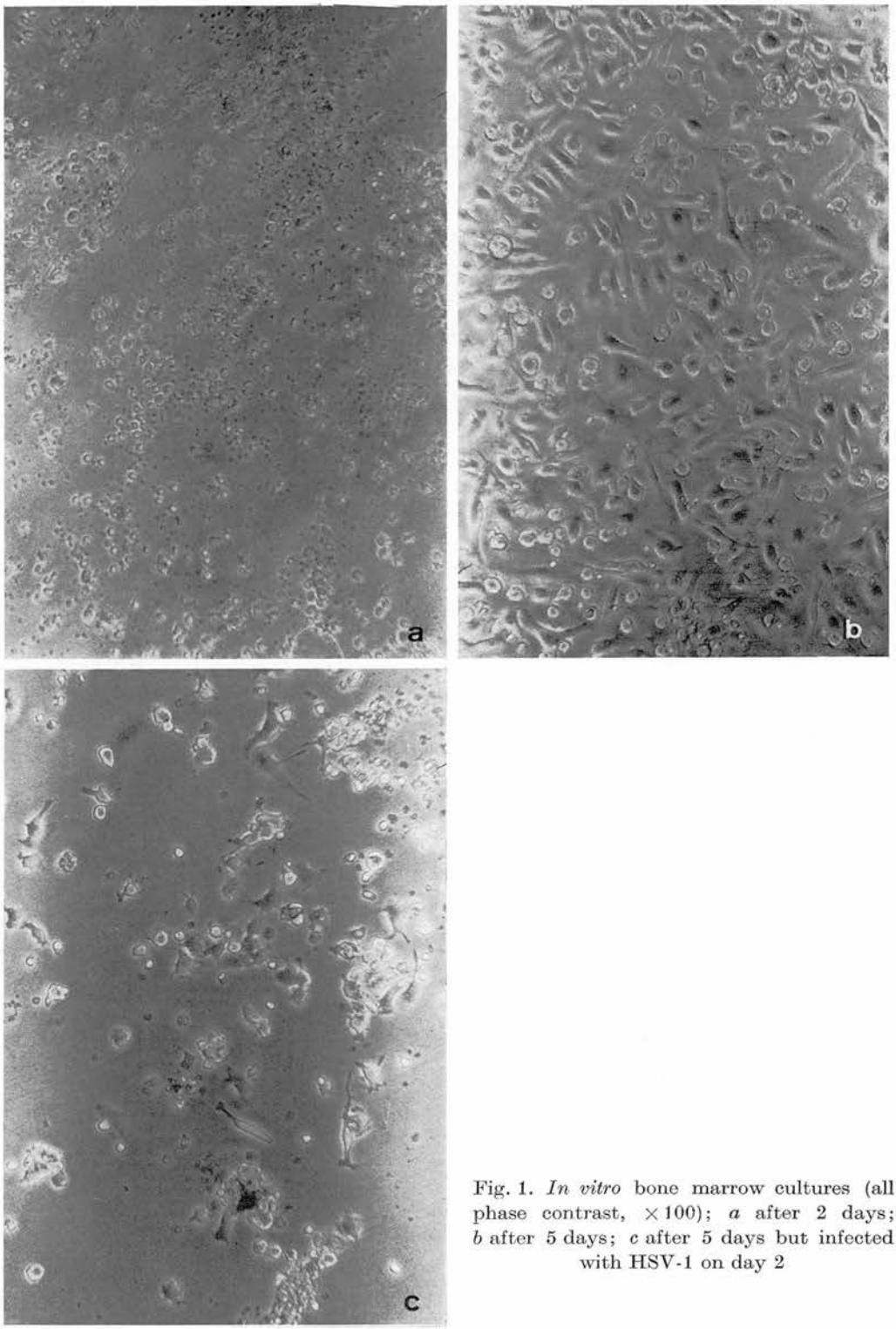

Fig. 1. *In vitro* bone marrow cultures (all phase contrast, ×100); *a* after 2 days; *b* after 5 days; *c* after 5 days but infected with HSV-1 on day 2

marked changes (Fig. 1 c). There was a reduction in cell numbers to around 10^5 per well 24 hours post-infection and this dropped further to around 8×10^4 per well 72 hours post-infection. If incubated for longer than 72 hours, the infected cultures gradually recovered with the adherent cells growing out into micro-colonies.

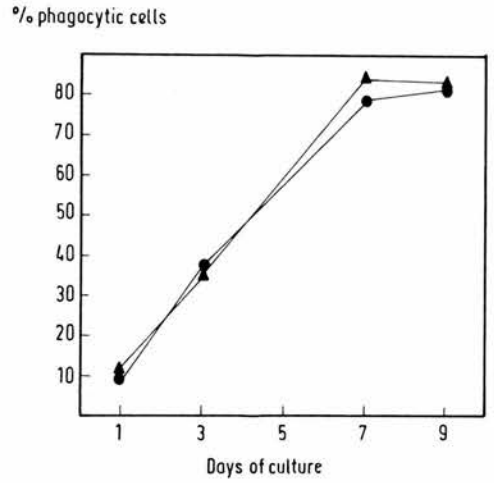

Fig. 2. Percentage phagocytic cells in bone marrow cultures incubated for 0, 2, 6 and 8 days *in vitro* before infecting with HSV-1 and assayed 24 hours later (▲——▲ infected with HSV; ●——● mock-infected controls)

These studies were all done using the same amount of virus per well which gave a multiplicity of infection of 1 on day 0 and about 5 at other time points. Infection with one-tenth this quantity of virus did not result in any morphological differences over 7 days compared to uninfected cultures.

Phagocytosis

At 24 hours post-infection, at least 100 cells in each of 4 wells were counted to find the percentage capable of phagocytosing sensitized CRBC. The results are shown in Fig. 2. No difference was apparent between infected and uninfected cultures at any time.

Virus Yields

These are illustrated in Fig. 3. There was no indication of productive infection at any time. The experiment was repeated 3 times with the same result. Infection at a multiplicity of 0.1 instead of 1 gave virus yields consistently one-tenth that normally produced.

Immunofluorescence

The monoclonal antibody, T181, and the polyclonal rabbit antiserum were able to detect HSV antigens in Vero cells from 2 hours post-infection.

The fluorescence in both cases was predominantly in the form of nuclear granules. By 6 hours post-infection, there was overall nuclear fluorescence and by 24 hours the whole cell was fluorescent.

The antibody preparations were used on the bone marrow cells after they had been in culture for 0 to 8 days and had been fixed at 0, 1, 4 and 24 hours post-infection. No viral antigens were detected at any time.

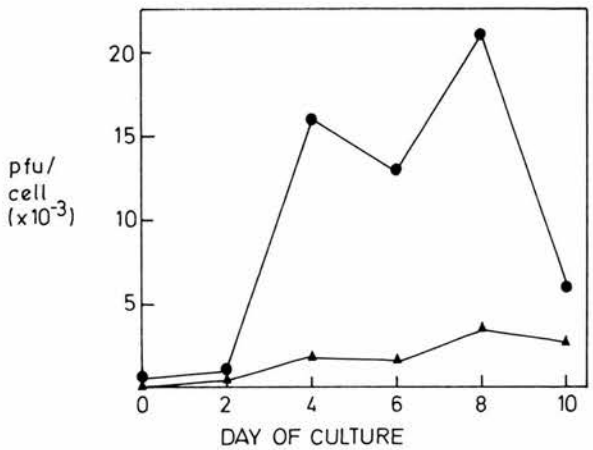

Fig. 3. Number of plaque-forming units/cell in bone marrow cultures infected with HSV-1 after being in culture for 0, 2, 4, 6, 8 and 10 days (●——● at 1 hour post-infection; ▲——▲ at 24 hours post-infection)

An infectious centre assay indicated that only 0.2 per cent bone marrow cells adsorbed HSV-1 on day 0. This rose to a level of 1 per cent after the cells had been in culture for 6 days (data not shown).

Fc Receptor Assays

The percentage of cells with Fc receptors was assayed during 7 days of *in vitro* culturing and the effect of HSV-1 measured 24 hours post-infection. This was repeated 3 times and the results of 1 representative experiment are shown in Fig. 4. The standard error for each point was less than 5 per cent of the mean, except for the first point. The cells obviously underwent differentiation during culture; no difference was found in Fc receptor expression between infected and uninfected cells. In one case cells were trypsinized (0.1 per cent trypsin for 30 minutes at 37° C) immediately before analysis to determine whether any virally induced trypsin-sensitive Fc receptors were present, but none was found.

MHC Class II Antigens

The final analysis was of MHC Class II antigen expression during the culture period and the effect of HSV-1 on this measured 24 hours post-

infection. The experiment was repeated 3 times and at least 500 cells were examined on each coverslip. The percentage showing positive staining from one representative experiment is shown in Fig. 5. The standard error for each point was less than 5 per cent of the mean. Initially the bone marrow cells had no detectable MHC Class II antigens but these were generated during culture until 50 per cent were positive. When the cells were infected with HSV-1, a consistently higher level of expression was seen compared to the uninfected cultures.

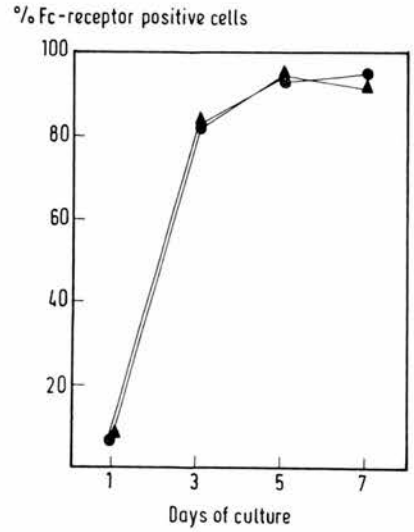

Fig. 4

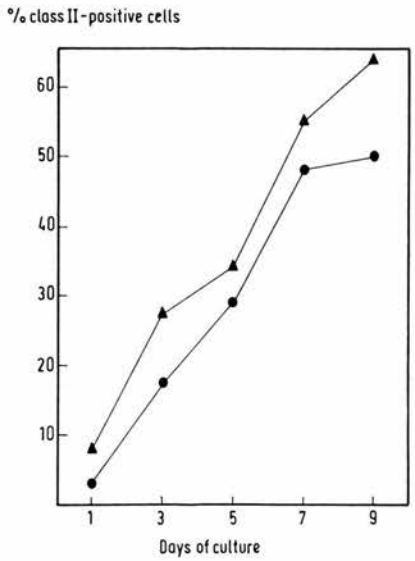

Fig. 5

Fig. 4. Percentage Fc receptor-positive cells in bone marrow cultures incubated for 0, 2, 4, 6 and 8 days *in vitro* before infecting with HSV-1 and assayed 24 hours later (▲——▲ infected with HSV; ●——● mock-infected controls)

Fig. 5. Percentage MHC class II-positive cells in bone marrow cultures incubated for 0, 2, 4, 6 and 8 days *in vitro* before infecting with HSV-1 and assayed 24 hours later (▲——▲ infected with HSV; ●——● mock-infected controls)

Discussion

By nonspecific esterase staining, Fc receptor expression and phagocytic ability, the bone marrow cells used here represent a culture of relatively pure macrophages which undergo maturation and proliferation *in vitro* in the presence of L-cell conditioned medium. Bone marrow derived macrophages are more homogeneous than peritoneal exudate cells and recently have been used as targets for the replication of mouse hepatitis virus type 3 (15). It was of interest to find out if HSV-1 had the same kind of interaction with these cells as has been found for peritoneal macrophages and whether this association changed with maturation during *in vitro* culturing. It has

already been reported that bone marrow macrophages from susceptible DBA/2 mice were able to support replication of HSV-1 after preculturing for 7 days, but to a lesser extent (one-tenth) than peritoneal macrophages from the same source (1). MORAHAN *et al.* (12), using DNA hybridization techniques, recently showed that viral DNA synthesis occurred in infected bone marrow macrophage cultures but no evidence of infectious virus production was obtained. The cells showed marked cytopathic effects.

In this study bone marrow macrophages from C3Hf Bu/Kam mice were cultured *in vitro* and infected with a clinical strain of HSV-1. This strain of mouse is relatively resistant to infection with the HSV-1 strain used, 10^7 PFU being required to induce paralysis and subsequent death in 90 per cent of female animals (aged 6—8 weeks) injected intraperitoneally. Injections of 3×10^6 PFU killed 1 out of 10 6—8 week old mice but 5 out of 5, 16 day old mice. Resistance to HSV-1 in various strains of mice is thought to be immunologically mediated and is not coded for in the major histocompatibility region (8).

The morphology of the bone marrow cells, which had been in culture for more than 2 days altered after infection with HSV-1. However yields of infectious virus indicated that the cells were not permissive. Indirect immunofluorescence using antibodies capable of detecting viral antigens induced 2 hours post-infection was negative, and an infectious centre assay showed adsorption of virus to only a few cells in the cultures. It is possible that these represent a small subpopulation of macrophages, below the limit of detection by immunofluorescence, which are capable of, at least, abortive infection. Subpopulations of peritoneal macrophages separated on the basis of size have not shown preferential permissiveness for HSV-1 replication, thus far (2, 11). However in the study here the majority of the bone marrow cells were found to be resistant to infection and the morphological changes noted may have been due to some modifying effect of the virus preparation. The stock of virus used probably includes non-infectious virus particles as well as infectious virus which could affect, for example, cell membranes, especially as the macrophages become more differentiated. In support of this, the adherent cells remaining 72 hours after infection began to grow and divide, and secondly, infecting at a multiplicity one-tenth lower than standard removed most of the differences noted between infected and uninfected cultures.

Three parameters, phagocytic ability, Fc receptor expression and MHC Class II expression were examined 24 hours post-infection. The first two were unaltered compared to the uninfected controls, but MHC Class II expression was increased at all time points. MHC Class II antigen expression at the cell surface is directly implicated in antigen presentation by non-lymphoid cells to specific T lymphocytes of the helper/inducer subsets. Thus a cell which does not express such antigens cannot act as an antigen presenting

cell during the immune response. It is known that MHC Class II antigen expression on macrophages can be modulated by several factors including activation state and interferon (16).

It is possible that the presence of HSV may induce interferon production in the bone marrow cultures which would increase expression of MHC Class II antigens on the cell surface. This hypothesis is currently being tested. *In vivo* this may have wide implications for the generation of immune responses to HSV, particularly at local sites of infection. Here high levels of immunity are thought to be important for viral clearance, and these may be induced by increasing the number of potential antigen presenting cells at the site by increasing MHC Class II antigen expression.

Acknowledgements

The authors are indebted to Graeme Dougherty for help with the immunoperoxidase stain.

We thank the Medical Research Council of Great Britain for financial support.

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Authors' address: Dr. Sarah Howie, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH 8 9AG, United Kingdom.

Received November 16, 1984

Printed in Austria

Exposure to Low-Dose Ultraviolet Radiation Suppresses Delayed-Type Hypersensitivity to Herpes Simplex Virus in Mice

Sarah Howie, Ph.D., Mary Norval, Ph.D., and Jean Maingay, B.A.
Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, Scotland

Ultraviolet B (UVB) radiation is reported to induce a defect in epidermal antigen presentation which leads to specific suppression of the delayed-type hypersensitivity (DTH) response to trinitrochlorobenzene. We have used a similar system to examine the murine DTH response to herpes simplex virus type 1 (HSV-1). Mice irradiated with 96 mJ/cm^2 UVB on shaved dorsal skin 3 days before s.c. injection of live HSV-1 in the flank showed 54–92% suppressed DTH responses to challenge with inactivated virus compared with nonirradiated control animals. If irradiation took place 7 days before inoculation with virus, some suppression of DTH occurred; if 14 days before, no suppression was found. The transient nature of the UVB response is further illustrated by the observation that irradiation with the same dose of UVB 5 h before, or 3 days after, inoculation with virus had no effect on DTH. Once induced, some degree of UVB suppression was found to persist for at least 3 months after irradiation. *J Invest Dermatol 86:125–128, 1986*

When herpes simplex virus (HSV) is inoculated s.c. into mice, the virus replicates locally reaching its highest titers between days 2–6 and is undetectable 10 days later [1]. HSV may then invade the sensory nerve endings in the local neurodermatome and establish latency in the sensory ganglia [2]. A delayed-type hypersensitivity (DTH) response can be detected by injection of virus or viral antigen into the ear pinna with measurement of ear swelling 24–48 h later. Delayed-type hypersensitivity is found 4 days after a primary infection, reaches a maximum at 8 days, and lasts for the lifetime of the animal [2]. It is under the control of the I region of the H-2 complex and plays a role in recovery from infection [3,4]. B cells that suppress the DTH response are found in the draining lymph node from 16 days onward [5]. The control of primary HSV infections is complex, but T cells seem to be important in early events, with evidence for both cytotoxic and DTH-mediating T cells being involved (reviewed in [6]).

Several groups, using a variety of systems, have shown that UV irradiation in vivo has a selective suppressive effect on the immune system [7,8]. Epidermal [9,10] and spleen [11] cells from UV-irradiated animals are deficient in antigen-presenting ability and antigen-specific T-suppressor lymphocytes may be generated as a result of this. UV radiation does not penetrate the skin and suppression is not due to local effects on the skin [12]. Although irradiation is known to affect the numbers and morphology of Langerhans cells, and to lead to increased production of epidermal cell-derived activating factor (ETAF) by keratinocytes, it has been shown recently that maximum suppression of DTH occurs at a different wavelength from the one directly affecting the Langerhans cells [13]. Thus, there may be release of an UV-induced photoproduct by epidermal cells which then interacts with Langerhans cells and suppresses their capacity to present antigen.

In the study reported here, infection with an epidermal pathogen, HSV-1, has been used in a mouse model. Measurement has been made of the DTH response to this virus and the effect of UV radiation on this parameter quantitated.

MATERIALS AND METHODS

Animals Female C3HfBu/Kam mice, bred and maintained in the departmental animal house, were used when 10–16 weeks old.

UVB Irradiation Mice were irradiated under a bank of 2 Philips TL20W/12 sunlamps at a distance of 30 cm. These bulbs had an output in the UVB range between 270–350 nm with a peak output at 310 nm. Measurement of output was made inside the perspex box, described below, using an Industrial Light Research Radiometer IL700A with an actinic optical assembly. The output was 0.8 J/m^2/s. Before irradiation, mice were shaved dorsally and their ears protected by black electrical tape [13]. When tested, this tape did not allow any penetration of UVB radiation. Mice were irradiated in a 5-chambered perspex box with deep walls and no lid, such that they could not turn around or over.

Virus The virus used was a plaque-purified clinical isolate of HSV-1, the DNA of which has been analyzed using 3 restriction endonucleases. The virus was passaged in vitro in Vero cells. Aliquots were stored at −70°C and sonicated for 60 s in an ultrasonicating water bath immediately before use. The number of plaque-forming units (pfu) of infectious virus present per milliliter of cell suspension was determined on Vero monolayers with 0.25% sea plaque agarose (FMC Corporation) in the overlay. Infectious virus was inactivated by UVB irradiation for 60 min. The stock preparation of virus used throughout had a titer of 10^{10} pfu/ml which was equivalent to 10^3 pfu per Vero cell. In mice, an i.p. injection of 10^7 pfu of this virus strain induced paralysis and death in 90% of animals.

1-chloro-2,4,6-Trinitrobenzene 1-chloro-2,4,6-Trinitrobenzene, obtained from BDH Chemicals Ltd., Poole, England, was made up to 5% (w/v) in acetone immediately before use.

Manuscript received April 18, 1985; accepted for publication August 22, 1985.
Supported by the Medical Research Council of Great Britain.
Reprint requests to: Sarah Howie, Ph.D., Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland.

Abbreviations:
APC: antigen-presenting cells
DTH: delayed type hypersensitivity
HSV: herpes simplex virus
PBS: phosphate-buffered saline
pfu: plaque-forming units
TNCB: 1-chloro-2,4,6-trinitrobenzene
UVB: ultraviolet B

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DTH Assay The principle of the assay was identical whether HSV-1 or TNCB was used as the sensitizing agent.

TNCB Assay: The abdomens of mice were shaved and then painted with 0.1 ml 5% TNCB in acetone or with acetone alone. Five to 8 days later, the mice were individually marked and their ear thicknesses were measured using a micrometer gauge (Draper Precision Instruments, Japan). They were then challenged by painting 15 μl of 5% TNCB in acetone onto both sides of each ear pinna. Twenty-four hours later, the ear thicknesses were again measured and the increase in ear thickness calculated. A mean of the 2 measurements from each mouse was taken before the mean and SE for each experimental group was calculated.

HSV-1 Assay: Mice were inoculated s.c. in the flank with 10^7 pfu of infectious virus or 10^4 uninfected sonicated Vero cells in phosphate-buffered saline (PBS). Eight days later, they were individually marked and their ear thicknesses were measured as above. They were then injected with 10 μl of PBS containing 10^6 pfu UV-inactivated HSV-1 in both ear pinnae. Twenty-four hours later, the ear thicknesses were again measured and the increase in ear thickness calculated. A mean of the 2 measurements from each mouse was taken before the mean and SE for each experimental group were calculated.

UVB-Induced Suppression of DTH To determine the suppressive effect of UVB on generation of DTH, mice were irradiated as described above 3 days prior to initial sensitization with TNCB or HSV-1 [13]. The net increase in ear thickness of control and UVB-irradiated mice was taken as the difference between the mean ear swelling of sensitized and unsensitized mice. Percentage suppression was calculated according to the formula:

$$\% \text{ suppression} = 100 - \frac{100 \times \text{net increase in UV-irradiated mice}}{\text{Net increase in control mice}}$$

as described by Noonan et al [13].

RESULTS

Calibration of the Suppressive Effect of UVB on DTH Responses Mice were irradiated with increasing amounts of UVB 3 days prior to sensitization with TNCB (UV experimental group) or acetone alone (UV control group) and were compared with control and experimental groups that had not been exposed to UVB. Table I shows that 20-min irradiation with UVB (equivalent to 96 mJ/cm^2) caused 60% suppression of the DTH response. This dose of UVB did not give rise to any observable burning of the skin. Mice irradiated for 30 min did demonstrate burning on the skin of their backs and the decreased suppression of the DTH response seen in this group may be explained by an overriding generalized inflammatory reaction to the epidermal damage. For this reason, it was decided to use 20-min irradiation with UVB in all subsequent experiments.

Table I. UVB-Induced Suppression of DTH to TNCB

Sensitized with	UVB Irradiation	24-h Increase in Ear Thickness (units × 10^2 mm ± SEM[a])	% Suppression of DTH
Acetone	Nil	8.2 ± 1.14	—
TNCB	Nil	27.3 ± 2.01	—
TNCB	10 min	20.2 ± 1.74[b]	37
TNCB	20 min	15.8 ± 0.84[b]	60
TNCB	30 min	19.0 ± 1.94[b]	44

All mice were challenged with 15 μl TNCB in acetone on both sides of both ear pinnae.

[a]Five mice per group.

[b]Significantly different from the experimental group sensitized with TNCB without prior UVB exposure, $p < 0.001$ in all cases (Student's *t*-test).

Induction of Specific DTH to HSV-1 Mice were inoculated with live HSV-1 or an equivalent amount of uninfected Vero cell s.c. in the flank. As described in *Materials and Methods*, the 24-h DTH response was determined after challenge with inactivated virus (cultured in Vero cells) on day 8. The induction of DTH to HSV-1 is shown in Table II, which also indicates that the DTH to the virus is specific in nature and directed against HSV-1 antigens rather than Vero cell antigens.

DTH to HSV-1 is Suppressed by UVB Treatment To examine the effect of UVB pretreatment on the generation of DTH to HSV-1, mice were shaved dorsally and UV-irradiated for 0 or 20 min as above. Three days later, they were inoculated with either 10^4 sonicated Vero cells or 10^7 pfu of HSV-1 as above. The 24-h DTH response to challenge 8 days later with 10^6 UV-inactivated pfu of HSV-1 was determined as above. Table III shows that this single exposure to a suberythemal dose of UVB irradiation induced 54–92% suppression of the DTH response to HSV-1

UVB-Induced Alteration in the Generation of DTH is Transient and Short-Term in Nature To determine the relationship between the time of exposure to UVB and the time of sensitization with virus, mice were irradiated at several time points before and after infection with HSV-1. Fig 1 shows that mice irradiated 14 days before sensitization to virus demonstrated no suppression of DTH, whereas mice irradiated 7, 3, and 2 days before sensitization were suppressed by 40, 70, and 82%, respectively. Irradiation on the same day as (5 h before) or 3 days after, sensitization to virus had no effect on DTH responses. This indicates that the UVB-induced generation of DTH suppression is transient, short-term in nature, and that it takes up to 2 days to be effective.

However, in contrast to this result, mice that had been UVB suppressed for DTH to HSV-1 remained suppressed for as long as tested. This was up to 3 months after UV-induced DTH suppression had been demonstrated (data not shown). Thus the suppressed state, once generated, is not transient.

DISCUSSION

Knowledge of the immunologic mechanisms that control HSV infections is crucial to an understanding of HSV pathogenesis. This is important both for the prevention of spread of primary disease and for attempts to modify the pattern of subsequent episodes of epidermal recrudescence.

We have examined how a well-characterized in vivo immune response, DTH, to experimental HSV-1 infection [4,5,14] is altered by UV irradiation, a stimulus known to affect both viral pathogenesis [15] and the correct functioning of the immune system itself [9,13].

Table II. Induction and Specificity of DTH to HSV-1

Sensitized with	Challenged with	Increase in Ear Thickness (units × 10^{-2} mm ± SEM)
Vero	HSV-1	6.8 ± 0.92 (n = 5)[a]
HSV-1	HSV-1	17.7 ± 0.5 (n = 5)
Vero	TNCB	9.5 ± 0.92 (n = 5)
HSV-1	TNCB	8.1 ± 0.42 (n = 5)
Acetone	TNCB	9.3 ± 1.0 (n = 5)
TNCB	TNCB	23.7 ± 0.68 (n = 6)
Acetone	HSV-1	4.9 ± 0.52 (n = 5)
TNCB	HSV-1	5.4 ± 0.76 (n = 5)

Mice sensitized on day 0 with 0.1 ml 5% TNCB in acetone, acetone alone, 10^7 pfu infectious HSV-1 grown in Vero cells or 10^4 Vero cells alone (see *Materials and Methods* for inoculation details).

Mice challenged on day 8 with either 15 μl 5% TNCB in acetone on both sides of each ear pinna or by injection of 10^6 pfu UV-inactivated HSV-1 into each ear pinna.

[a]n = Number of mice.

Table III. Suppression of DTH to HSV-1 by UVB Irradiation 3 Days Before Sensitization

UVB Irradiation (min)	Sensitized with	Increase in Ear Thickness (units × 10^{-2} mm ± SEM)		
		Experiment 1	Experiment 2	Experiment 3
0	10^4 Vero	3.4 ± 0.7 (n = 5)[a]	3.53 ± 0.36 (n = 8)	2.9 ± 0.51 (n = 5)
0	10^7 HSV-1	13.31 ± 0.84 (n = 5)	9.3 ± 0.73 (n = 5)	14.0 ± 0.90 (n = 6)
20	10^4 Vero	1.45 ± 0.65 (n = 5)	3.75 ± 0.53 (n = 6)	3.5 ± 0.65 (n = 8)
20	10^7 HSV-1	2.2 ± 1.02 (n = 5)	4.4 ± 0.47 (n = 6)	8.6 ± 0.92 (n = 5)
% suppression of DTH		92%	88%	54%

All mice challenged on day 8 (after sensitization) with 10^6 UV-inactivated pfu HSV-1 in both ear pinnae.
[a] n = Number of mice.

The results described demonstrate that exposure to a single suberythemal dose of 96 mJ/cm² UVB irradiation at a critical time (2–7 days) prior to s.c. inoculation with infectious virus suppressed the DTH response to HSV-1. Significant suppression persisted for as long as tested (1 month) after UVB irradiation. No suppression was generated if virus was injected immediately, or 3 days prior to irradiation with the same dose of UVB. This suggests that skin exposed to UVB is altered over a period of time such that immune responses against antigens encountered up to 7 days subsequently, do not follow the "normal" pattern.

UVB irradiation is known to affect the antigen-presenting capacity of the skin [9,10] and other organs [11]. It also causes an alteration in antigen handling such that foreign antigens subsequently encountered induce the systemic generation of specific suppressor cells. This phenomenon has been described for contact sensitizing agents [13] and also for tumor antigens [7]. The murine skin is reported to contain 2 functional sets of antigen-presenting cells (APC), one sensitive to UVB irradiation and inducing positive immunity, and the other resistant to UVB and inducing specific suppressor cells [16]. The balance between these sets must be delicately controlled and any factor that alters this will have far-reaching effects on the immune response to antigens subsequently encountered. Recently, it has been reported that antibody responses to topically applied antigens are also suppressed by UV irradiation; in this case, failure to prime due to a defect in antigen presentation was the main mechanism postulated [17]. The effects of UV irradiation on the antibody responses to HSV-1 are currently being investigated.

As suppression was found to be long-lasting, it is unlikely to be due to a loss of APC from the epidermis as it is known that skin depleted of Langerhans cells by tape stripping is repopulated within 24–48 h [18] and that animals first exposed to an antigen 2 weeks after UVB irradiation have normal DTH responses [19].

Adoptive transfer experiments are in progress to characterize any population of immune cells induced by UVB irradiation. In addition, the effect of irradiation on the function of epidermal cells and other APC after HSV-1 infection is being investigated.

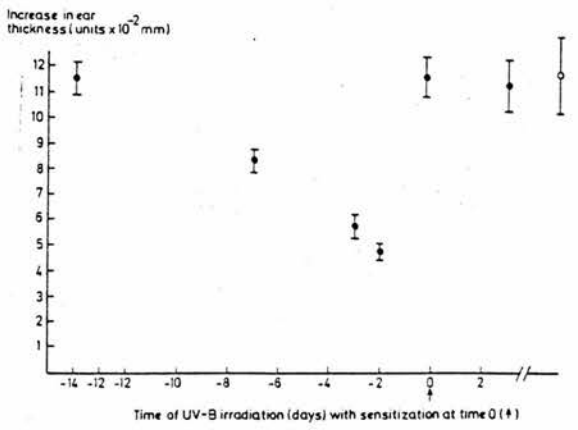

Figure 1. Kinetics of UVB induction of DTH suppression. HSV-1 (10^7 pfu) was used for sensitization and challenge was 8 days later with 10^6 UV-inactivated pfu HSV-1 in both ear pinnae. At least 5 mice were present each group and SEM for each point are shown. (The increase in ear ckness of an equivalent group of nonirradiated mice is shown, ○).

The authors are indebted to Drs. E. de Fabo and M. Fisher for their detailed advice about the UVB irradiations.

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Alterations in epidermal handling of HSV-1 antigens *in vitro* induced by *in vivo* exposure to UV-B light

S. E. M. HOWIE, M. NORVAL & J. P. MAINGAY *Department of Bacteriology, University of Edinburgh Medical School, Edinburgh*

Accepted for publication 20 September 1985

SUMMARY

In order to investigate the cellular interactions involved in the immune response to herpes simplex virus type 1 (HSV-1) in a murine model, an *in vitro* antibody induction system was developed. This comprised HSV-1-primed T cells from infected mice, trinitrophenol (TNP)-primed B cells from mice primed with TNP-coupled calf erythrocytes, and TNP-HSV-1 as antigen. When antigen-presenting cells (APC) were removed from the assay system, the induced antibody response disappeared but could be reconstituted by the addition of APC derived from the peritoneal cavity or skin of normal mice. Since HSV-1 is an epidermal pathogen, it was decided to investigate the role of skin APC in HSV-1 immunity. Skin APC from mice irradiated 3 days previously with a suberythemal dose of ultraviolet (UV)-B were found to have a decreased capacity to present HSV-1 antigen *in vitro*. However, the APC capacity of their peritoneal cells was unaffected. The reduction in APC capacity is not only a local effect at the irradiated site, as APC from mice exposed to UV-B with one ear protected by black electrical tape were equally affected in both ears.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is usually first encountered in childhood when it causes an epidermal infection of the mouth area with mild fever which is often not noticed. The virus replicates and is thought to travel up the sensory neurones to the trigeminal ganglion where it remains latent. In some individuals the virus is reactivated at intervals throughout life and may recrudesce causing a lesion (cold sore) at the epidermal site of the original infection. Recrudescence has been shown to be triggered by a variety of stimuli, including colds, fever, sunlight, menstruation, physical trauma and stress (reviewed in Mims & White, 1984).

The importance of the immune system in controlling primary and latent HSV-1 infection has been demonstrated both experimentally and in natural infections, and has been recently extensively reviewed (Rouse & Lopez, 1984; Wildy & Gell, 1985). However, the relative roles of the various facets of the immune response, specific and non-specific, humoral and cell-mediated, remain unclear.

The generation of any specific immune response to a thymus-dependent antigen such as HSV-1 (Burns, Billup & Notkins, 1975) initially requires antigen-presenting cells (APC). Epidermally encountered pathogens like HSV-1 are presumably processed and presented to specific T lymphocytes on the surface of APC, the Langerhans cells (LC), within the skin. LC have been shown experimentally to be susceptible to modulation by external factors such as epidermal tape stripping and UV-B light exposure. The former depletes skin of epidermal LC and Ia immunogenicity (Streilein, Lonsberry & Bergstresser, 1982). The latter alters APC function in the skin in such a way that suppressive immune responses are preferentially induced to both tumour (Fisher & Kripke, 1978) and skin-sensitizing antigens (Noonan *et al.*, 1984). Both epidermal tape stripping and exposure to UV-B light are stimuli that can cause recrudescence of HSV-1 in murine models (Harbour, Hill & Blyth, 1983). Thus, a stimulus that affects the generation of immunity to epidermally encountered antigens also affects the pathogenesis of an epidermal viral infection.

We have reported elsewhere (Howie, Norval and Maingay, 1985) that UV-B light suppresses the delayed type hypersensitivity (DTH) response to HSV-1 in a mouse model. In order to investigate further the role of epidermal APC in HSV-1 infections, an *in vitro* assay for antibody production has been developed. This is based on a system described previously for measuring responses to tumour-specific antigens (Howie & McBride, 1982), and allows examination of the roles of APC

Abbreviations: APC, antigen-presenting cells, BBS, borate-buffered saline; C, complement; CRBC, calf erythrocytes; EBM, Eagle's based medium; ESC, ear skin cells; FCS, fetal calf serum; HSV-1, herpes simplex virus type 1; LC, Langerhans cells, NCS, newborn calf serum; NW, nylon wool passed; PBS, 0·01 M phosphate-buffered saline, pH 7·2; PC, peritoneal cells; PFC, plaque-forming cell; PFU, plaque-forming units; SRBC, sheep erythrocytes; T_h, T helper; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TNP, trinitrophenol; UV, ultraviolet.

Correspondence: Dr S. E. M. Howie, Dept. of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

and virus-specific T helper (T_h) cells in the generation of antibody production.

Using this system in the study of the immune response in mice after infection with HSV-1, it has been possible to characterize virus-specific T_h cells and APC from several sites and to examine the effect of UV-B light on APC capacity. The implications of the results obtained for understanding pathogenic mechanisms in HSV infections are discussed.

MATERIALS AND METHODS

Mice

C3HfBu/Kam(H-2-^{k}) female mice aged between 8 weeks and 20 weeks were used throughout. The mice were bred and maintained in the departmental animal house.

Reagents

2,4,6-Trinitrobenzene sulphonic acid (TNBS), glycyl glycine and DNase were purchased from Sigma Chemical Co., Poole, Dorset; Percoll from Pharmacia, Uppsala, Sweden; fetal calf serum (FCS) from Gibco Biocult Ltd, Paisley, Renfrewshire; RPMI-1640 from Flow Laboratories Ltd, Irvine, Ayrshire; 10 × Eagle's Basal Medium with Earle's salts (EBM), newborn calf serum (NCS), L-glutamine and trypsin from Imperial Laboratories Ltd, Salisbury, Wiltshire.

Sera

Monoclonal rat anti-Thy 1·2 (30·H·12) antibody was the kind gift of Dr H. S. Micklem, Department of Zoology, University of Edinburgh; monoclonal anti-Ia^k (TIB 93) (Oi *et al.*, 1978) was obtained from the ATCC; monoclonal anti-H-2K^k (HB5) was the generous gift of Dr W. H. McBride, Department of Radiation Oncology, UCLA Centre for Health Sciences, Los Angeles, CA; monoclonal antibody YE236 against HLA-DR which cross-reacts with Ia^d but not Ia^k (Brickel *et al.*, 1981) was the kind gift of Professor I. McConnell, Department of Veterinary Pathology, Edinburgh University; ATH anti-ATL (anti-Ia^k) serum was purchased from Mercia Brocades Ltd, Weybridge, Surrey; rabbit anti-mouse IgG enhancing serum was prepared by repeatedly immunising a rabbit with purified mouse immunoglobulin as described previously (Howie & McBride, 1982).

Complement

Serum from normal outbred guinea-pigs as a source of complement was absorbed with either sheep erythrocytes (for PFC assays) or mouse spleen cells (for cytotoxicity).

Erythrocytes

Sheep erythrocytes (SRBC) from sheep No. R207 were the kind gift of Mr C. Burrells, Moredun Research Institute, Edinburgh. Calf erythrocytes (CRBC) were purchased from Tissue Culture Services, Slough, Berks.

Virus

A clinical isolate of HSV-1 which had been plaque-purified and whose DNA had been analysed by restriction enzymes was used throughout. The virus was passaged *in vitro* in Vero cells grown in EBM containing 5% NBS, 100 i.u./ml penicillin, 200 μg/ml streptomycin and 2 mM L-glutamine (complete EBM). Infectious virus was titrated as plaque-forming units (PFU) on monolayers of Vero cells overlaid with complete EBM containing 0·25% Seaplaque agarose (FMC Corporation).

Trinitrophenylation of HSV-1

Virus, grown in Vero cells, was harvested from the culture supernatant when the cytopathic effect was well advanced and concentrated by precipitation with 50% saturated $(NH_4)_2SO_4$. It was purified by caesium chloride density gradient centrifugation, pooling the fractions with density 1·27–1·295 g/ml. The purified virus was dialysed for 48 hr against borate-buffered saline, pH 8, (BBS) at 4°. The protein content of the virus solution was determined by the Lowry technique. A solution of 15 mg/ml TNBS in BBS was prepared and was added to the virus solution at a concentration of 25 μg/mg protein. The mixture was stirred for 1 h at room temperature in the dark and then dialysed for 24 hr at 4° against several changes of Dulbecco's phosphate-buffered saline, pH 7·2 (PBS). The trinitrophenylated virus particles were examined under the electron microscope and seen to be still enveloped. One hundred μg/ml virus protein was estimated to be roughly equal to 1(virus particles per ml.

Trinitrophenylation of erythrocytes

Trinitrophenylated SRBC and CRBC (TNP-SRBC and TNP-CRBC) were prepared as previously described (Howie & McBride, 1982).

Immunization of mice

TNP priming. Mice were injected intraperitoneally with 0·1 ml of 0·5% TNP-CRBC on two occasions at least 4 weeks apart. Spleens were used as a source of TNP-primed B cells at least 4 weeks after the last injection.

Virus inoculation. Mice were immunized with a variety of PFU of live HSV-1 by a variety of parenteral routes (see Table 2). When given an initial intraperitoneal dose of 10^7 PFU infectious virus, approximately 90% of C3HfBu/Kam mice aged 5–6 weeks developed neurological symptoms within 5 days and subsequently died.

UV-B treatment

Mice were exposed to 96 mJ/cm^2 UV-B irradiation. This was a suberythemal dose and was equivalent to 20 min under two Philips TL20W/12 bulbs in the UV-B apparatus in the department animal house.

Cell preparations

Peritoneal cells were obtained by lavage of the peritoneal cavity with 5 ml PBS.

Ear cells were prepared by a modification of the method of Stingl *et al.* (1978). Ears from normal or UV-B-exposed mice were split and incubated in 0·5% trypsin for 1 hr at 37°. They were removed from the trypsin, washed by immersion in PBS and vigorously mixed on a rotamixer in PBS containing 0·05% DNase. The resulting cell suspension was layered onto a 20/50% discontinuous Percoll gradient and spun at 400 ***g*** for 20 min. Subcellular debris floated on top of the 20% layer and cells were found at the 50% interface and in the pellet. The cells in the starting population were 1–3% Ia+ve by immunoperoxidase staining and 3–5% Fc receptor +ve by rosette formation with

ntibody-coated CRBC. The cells on the 50% interface (25% of ıe total cells) were 10–17% Ia+ve and 15–20% Fc receptor -ve, while the cells in the pellet had only background levels of oth Ia+ve and Fc receptor +ve cells (<1%). The 50% Percoll ıterface yielded $1{\cdot}5 \times 10^5$ cells per mouse ear and was used as ıe source of ear antigen-presenting cells.

Spleen cells were prepared by homogenization of aseptically ɔmoved spleens. Viable cells were determined by trypan blue xclusion. Various subpopulations of spleen cells were separted out: (i) spleen cells were enriched for T cells by passage over ylon wool columns (Julius, Simpson & Herzenberg, 1973); (ii) ɔleen cells were depleted of T cells by treatment with anti-Thy-1 nd complement, leaving a population of B cells and APC; (iii) -depleted spleen cells were incubated with carbonyl iron to ınctionally remove APC as described previously (Howie & eldmann, 1978). Cells, 5×10^7, were mixed with 0·1 g carbonyl on at 37° for 1 hr. Cells that had taken up iron particles were ɔmoved from the suspension with a magnet.

ntibody-forming cell cultures

ıll cultures were incubated in 200-μl volumes and quadrupliated in 96-well microtitre plates. The medium used throughout ıas RPMI-1640 containing 5% FCS, 5×10^{-5} M 2-mercaptoethnol, 100 i.u/ml penicillin, 200 μg/ml streptomycin and 2 mM L-lutamine (complete RPMI). Anti-Thy-1 and complement-reated spleen cells, or the same depleted of APC, were plated at concentration of 5×10^5 viable cells per well in the presence of 3 ıg/ml TNP-HSV or 2×10^7 TNP-CRBC as antigen. T_h, 5×10^4 ɔer well from appropriate sources, were added, and APC as equired. The cultures were incubated for 4 days at 37° in a ıumidified 5% CO_2 incubator. At the end of this time, the quadruplicate cultures were harvested, pooled, washed and the ıumber of direct and indirect PFC in the cultures determined by Cunningham plaque-forming cell (Cunningham & Szenburg, 968) assay in the presence of complement and enhancing erum. The results were calculated as the number of PFC ounted with SRBC as indicator cells subtracted from those ounted using TNP-CRBC and are expressed as the number of PFC/10^6 input TNP-primed spleen cells.

RESULTS

TNP-HSV-1 acts as antigen in vitro

Mice were inoculated with 10^6 PFU HSV-1 in the ear pinna, and 4 weeks later with 10^7 PFU intraperitoneally. Eight weeks after he second injection, spleens were removed, homogenized and passed over nylon wool columns to yield a population of T ymphocytes (>95% Thy-1 +ve by immunofluorescence). The T cells from the HSV-1-infected mice were mixed in a 1:10 ratio vith anti-Thy-1 and complement-treated spleen cells from mice primed with TNP-CRBC. A range of doses of TNP-HSV-1 were itrated into the cell mixtures as antigen. It can be seen from Fig. that TNP-HSV-1 induced good PFC responses at 1–10 μg/ml of culture. In all subsequent experiments 3 μg TNP-HSV-1 per ml of culture were used.

The helper cells found in HSV-1-primed spleens are specific T cells

Table 1 demonstrates that the helper cells involved in generating anti-TNP PFC in the presence of TNP-HSV were found in whole spleen and in nylon wool-passed spleen cells, but not in anti-Thy-1 and complement-treated spleen cells of HSV-1-infected mice. The cells did not act as helper cells when TNP-CRBC was used as antigen. Thus, HSV-1-specific T_h cells (T_h-HSV-1) were present in the spleens of HSV-1-infected mice.

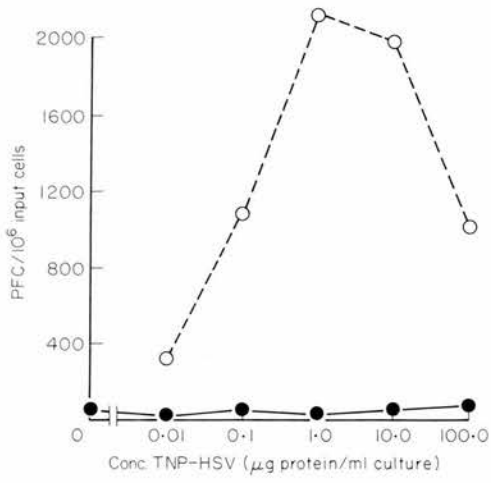


Figure 1. Trinitrophenylated HSV-1 stimulates antibody formation by T-cell depleted TNP-CRBC-primed spleen cells in the presence of nylon wool-passed spleen cells from HSV-1-infected mice. For culture conditions, see 'Materials and Methods'. (○ – – – ○) PFC in the presence of nylon wool-passed HSV-1-primed spleen cells plus anti-Thy-1 and C-treated TNP-CRBC-primed spleen cells and TNP-HSV-1 as antigen. (●——●) PFC generated by anti-Thy-1 and C-treated TNP-CRBC-primed spleen cells in the presence of TNP-HSV-1 as antigen.

Table 1. HSV-1 helper cells are T cells and antigen-specific

Antigen	Helper cell source	PFC/10^6 cells*
None	None	80
TNP-CRBC	None	120
TNP-CRBC	NW TNP-CRBC spleen	1300
TNP-CRBC	NW HSV-1 spleen†	150
TNP-HSV-1	None	60
TNP-HSV-1	NW TNP-CRBC spleen	10
TNP-HSV-1	Whole HSV-1 spleen	860
TNP-HSV-1	NW HSV-1 spleen	1520
TNP-HSV-1	Anti-Thy-1 and C-treated HSV-1 spleen	190

* Number of PFC generated by $2{\cdot}5 \times 10^6$/ml TNP-CRBC primed, anti-Thy-1 and C-treated spleen cells reconstituted with $2{\cdot}5 \times 10^5$/ml helper cells as shown in the presence of either 10^7/ml TNP-CRBC or 3 μg/ml TNP-HSV-1 as a source of antigen.

† Mice inoculated with 10^6 PFU infectious HSV-1 into the ear pinna 4 months previously and with 10^7 PFU 3 months previously.

HSV-1-specific T_h cells are present in the spleens of mice inoculated with a variety of doses of virus by a variety of parenteral routes

Table 2 shows that mice inoculated with a variety of doses of HSV-1 by a variety of parenteral routes had good levels of T_h-

Table 2. HSV-1-specific T-helper cells are found in the spleens of mice infected by different routes

HSV-1 infections (PFU infectious virus injected)				
1st inj.	Interval 2nd inj.	Interval until use	PFC without T_hHSV-1*	PFC with T_HHSV-1†
10^6 ear pinna	24 days 10^7 i.p.	59 days	10	1970
10^6 ear pinna	24 days 10^7 ear pinna	83 days	60	1520
5×10^6 s.c. flank	20 days 5×10^7 i.p.	17 days	70	1450
3×10^4 ear pinna	8 days 10^6 UV‡ ear pinna	9 days	80	1250
10^6 i.p.	— —	31 days	30	910

* Number of PFC generated by $2{\cdot}5 \times 10^6$/ml TNP-CRBC primed, anti-Thy-1 and C-treated spleen cells in the presence of 3 μg/ml TNP-HSV-1 as a source of antigen.

† As above, but with the addition of $2{\cdot}5 \times 10^5$/ml nylon wool-passed spleen cells from HSV-1-infected mice.

‡ UV-B inactivated (1 hr exposure) virus used.

HSV-1 in their spleens as long as 4 months after initial exposure to virus. In all cases, the T_h were antigen-specific (data not shown).

The antibody response to TNP-HSV can be APC-depleted and reconstituted with APC from the peritoneal cavity and ear skin

It can be seen in Table 3 that incubation of anti-Thy-1 and complement-treated TNP-primed spleen cells with carbony iron depleted the response to TNP-HSV in the presence of T_h HSV-1. The antibody response was restored by the addition o APC derived from the peritoneal cavity or from ear skin. Th restoration can be blocked by pretreatment of the APC wit either polyclonal or monoclonal anti-Ia^k, but not by monoclor al anti-H-$2K^k$ antibody or monoclonal anti-Ia^d antibody.

Table 3. APC-depleted spleen cells can be reconstituted by cells derived from the peritoneal cavity and from ear skin; the reconstitution is blocked by anti-Ia^k antibodies

APC source*	Antibody pretreatment†	PFC/10^6 cells‡ (% reconstitution)§ Experiment 1	Experiment 2
None	None	40 (3)	170 (11)
3% PC¶	None	1170 (77)	1160 (73)
3% PC	1:10 ATH α ATL	370 (25)	ND**
3% PC	1:5 mcIa^d	1430 (95)	ND
3% ESC††	None	1470 (97)	1540 (97)
3% ESC	1:10 ATHαATL	410 (27)	ND
3% ESC	1:10 mcIa^k	ND	360 (23)
3% ESC	1:5 mcIa^d	1360 (90)	ND
3% ESC	1:2 mcH-2K^k	ND	1610 (100)
Controls ‡‡			
Undepleted + TNP-HSV-1		60	30
Undepleted + TNP-HSV-1 + T_h-HSV-1		1510	1590

* APC from normal mice.

† APC were incubated at 10^6/ml with the stated final concentrations of antibodies for 2 hr at 4°. The cells were then washed three times with 20 ml PBS and added to APC-depleted cultures. For details of antibodies, see 'Materials and Methods'.

‡ All cultures (except controls) consisted of $2{\cdot}5 \times 10^6$/ml APC and T-cell depleted TNP-CRBC primed spleen cells + 3 μg/ml TNP-HSV-1 + $2{\cdot}5 \times 10^5$/ml nylon wool-passed HSV-1-primed spleen cells.

§ PFC/10^6 cells expressed as a percentage of the number obtained in non-APC-depleted cultures (see controls).

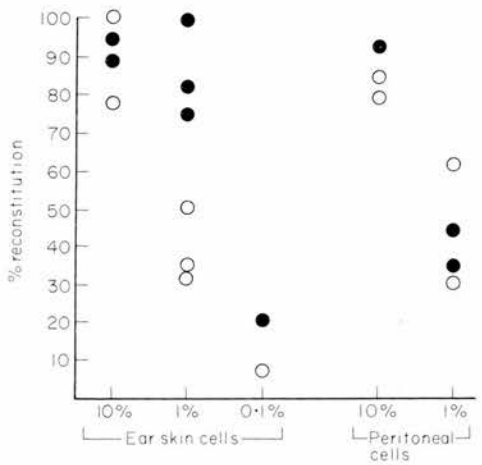
¶ PC, peritoneal cells.

** ND, not determined.

†† ESC, ear skin cells.

ıe APC function of ear skin cells but not of peritoneal cavity cells diminished by *in vivo* exposure to UV-B light

was previously shown that UV irradiation of mice with a berythemal dose of 96 mJ/cm² 3 days before (but not 5 hr :fore or 3 days after) inoculation with live HSV-1 suppressed e DTH response to HSV-1 (Howie, Norval & Maingay, 1985). order to examine this further, the APC function of ear skin lls that had been pre-exposed to UV-B 3 days before use *in tro* was tested and compared with the APC function of :ritoneal cells from the same animals. The results of three parate experiments are shown in Fig. 2. At the level of 10% ear



gure 2. All cultures consisted of $2{\cdot}5 \times 10^6$/ml APC and T-cell depleted NP-CRBC-primed spleen cells + 3 μg/ml TNP-HSV-1 + $2{\cdot}5 \times 10^5$/ml ylon wool-passed HSV-1-primed spleen cells. APC were obtained from ɔrmal (●) mice or mice UV-irradiated 3 days before use *in vitro* (○). he percentage reconstitution was calculated as the number of PFC/10^6 :lls expressed as a percentage number obtained in non-APC-depleted ıltures as detailed in Table 3. Each point represents one experiment.

:in cells in the test system, both irradiated and non-irradiated :lls could reconstitute the antibody response. However, at the :vel of 1% and 0·1% ear skin cells in the test system, there was iminished ability to present HSV-1 antigens if the mice had een UV-irradiated ($P = <0{\cdot}001$ by Student's *t*-test). The ınction of the peritoneal APC was not altered by UV radiation. This may suggest that there are fewer functional .PCs in irradiated skin. Limiting dilution assays are being erformed to test this.

:eduction in APC capacity of ear skin cells following *in vivo* UV-ł light exposure is not just a local effect

When mice were irradiated with UV-B light as above, but with ne ear protected by black electrical tape, the reduction in APC apacity was found in both the taped and untaped ears. The tape id not allow penetration of any UV light. The APC capacity of eritoneal cells from the same mice was unaffected. This xperiment was performed three times with the same results, and he data from one experiment are shown in Table 4. Taped and ntaped ears from non-UV-irradiated mice were also compared, nd there was no difference in their ability to present antigen *in itro* (data not shown).

Table 4. Reduction in APC capacity of ear skin cells following UV-B exposure is not solely a local effect

APC source	PFC/10^6 cells† (% reconstitution)‡
None	40 (3)
Normal PC 10%	1320 (87)
Normal PC 3%	1170 (77)
Normal PC 1%	930 (62)
UV-B PC 10%	1280 (85)
UV-B PC 3%	1180 (78)
UV-B PC 1%	860 (57)
Normal ESC 10%	1530 (100)
Normal ESC 3%	1470 (97)
Normal ESC 1%	1060 (70)
UV-B ESC taped 10%	1140 (75)
UV-B ESC taped 3%	490 (32)
UV-B ESC taped 1%	270 (18)
UV-B ESC untaped 10%	990 (66)
UV-B ESC untaped 3%	500 (33)
UV-B ESC untaped 1%	320 (21)
Controls	
Undepleted + TNP-HSV-1	60
Undepleted + TNP-HSV-1 + T_h-HSV-1	1510

* APC from peritoneal cavity and ear skin of normal mice and mice irradiated *in vivo* with 96 mJ/m² UV-B with one ear protected by black electrical tape (six mice per group).

† All cultures (except controls) consisted of $2{\cdot}5 \times 10^6$/ml APC and T-cell depleted TNP-CRBC primed spleen cells + 3 μg/ml TNP-HSV-1 + $2{\cdot}5 \times 10^5$ nylon wool-passed HSV-1-primed spleen cells.

‡ PFC/10^6 cells expressed as a percentage of the number obtained in non-APC-depleted cultures (see controls).

§ Response of non-APC-depleted cultures.

DISCUSSION

In vivo UV-B has been shown to deplete skin of LC (Toews, Bergstresser & Streilein, 1980) and to induce preferentially the formation of T suppressor cells to tumour antigens (Fisher & Kripke, 1978), contact sensitizing antigens (Noonan *et al.*, 1984) and photosensitizing antigens (Takigawa *et al.*, 1984). UV-B exposure has also been shown to deplete skin of APC function *in vivo* (Perry & Greene, 1982) and in subsequent *in vitro* assays (Stingl *et al.*, 1981), and to deplete LC of their surface markers (Aberer *et al.*, 1981).

It has recently been shown, however, that the alterations in epidermal density and morphology of LC after UV irradiation occur at a different wavelength from that which induces systemic suppression of contact hypersensitivity (Noonan *et al.*, 1984; Morrison, Bucana & Kripke, 1984). LC then may not be directly responsible for the suppression but may interact in some way with a UV-induced photoformat. Alternatively, Granstein, Lowy & Green (1984) have recently reported a novel skin APC in UV-B irradiated skin which is I-J +ve, UV-resistant, and may be the APC responsible for T-suppressor cell generation.

We have previously shown that DTH responses to HSV-1 *in vivo* can be suppressed by pre-exposure of mice to a suberythemal dose of 96 mJ/cm² UV-B 3 days before infection with virus. Exposure to the same dose of UV-B immediately before or 3

days after inoculation with live HSV-1 had no effect on DTH (Howie, Norval & Maingay, 1985). In accordance with the work of Noonan *et al.* (1984), who measured DTH to the skin-sensitizing agent TNCB, these results suggest that there is a critical time after exposure to UV-B light, during which the ability of the skin to handle antigen is altered. For this reason, we decided to investigate the effects of UV-B exposure on the ability of skin cells to present HSV-1 antigens in more detail.

The experiments described here show: (i) that skin cells from normal mice can present HSV-1 antigens in an *in vitro* antibody-induction system, (ii) that pre-exposure to UV-B 3 days before use *in vitro* diminishes the capacity of ear skin cells to present HSV-1 antigens, but does not alter the APC capacity of peritoneal cells from the same animals, and (iii) that the UV-B-induced reduction in APC capacity is not local to the site of irradiation.

Given the importance of the immune system in controlling HSV-1 infection, any stimulus that alters the immune response to epidermally encountered antigens may affect the pathogenesis of primary and possibly of recrudescent HSV-1 infections also. It is tempting to speculate that one factor which may determine the outcome of primary HSV-1 infection is the initial type of immune response generated. If the virus is encountered at a time when the APC capacity of the epidermis has been altered by some stimulus, the initial immune response to HSV-1 may not be wholly appropriate. It is not known what effect this might have on the interaction of HSV-1 with the host or, indeed, what effect this might have on the establishment of latency and the frequency of recrudescences. We are currently investigating these areas.

ACKNOWLEDGMENTS

S. E. M. Howie is a Senior Research Fellow of the Medical Research Council of Great Britain.

This work was supported by M.R.C. Grant No. G8218353CA.

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Two phenotypically distinct T cells ($Ly1^+2^-$ and $Ly1^-2^+$) are involved in ultraviolet-B light-induced suppression of the efferent DTH response to HSV-1 *in vivo*

S. E. M. HOWIE, M. NORVAL, J. MAINGAY & J. A. ROSS *Department of Bacteriology, University Medical School, Edinburgh*

Accepted for publication 1 April 1986

SUMMARY

We have previously demonstrated that the delayed-type hypersensitivity (DTH) response to herpes simplex virus type 1 induced by subcutaneous injection with live virus was suppressed by irradiating mice with a low dose (96 mJ/cm^2) of ultraviolet-B (UV-B) light 3 days before sensitization. In order to determine the nature of the suppression, cells from mice irradiated with UV-B before immunization with live virus were transferred to syngeneic animals that had been sensitized with live virus without prior UV-B exposure. Efferent suppression of DTH to HSV-1 was shown to be due to T lymphocytes, and two phenotypically distinct T cells were involved: an $Ly1^+2^-$ subset and an $Ly1^-2^+$ subset.

INTRODUCTION

Herpes simplex virus (HSV) is a major human epidermal pathogen. In some individuals the virus becomes latent and may recrudesce at varying intervals throughout life. The immune response has been shown to be important in determining the outcome of both primary and recrudescent infections, but which arms of the response are most crucial in the various stages of the host–virus interaction is not yet clear. It is known that cell-mediated immunity is of paramount importance in both clearing viral infection and controlling recurrences (reviewed in Nash, 1985). Any environmental stimulus that transiently depresses cell-mediated immunity generated at an epidermal site may have profound effects upon the nature of the host–virus balance initiated by primary exposure to the virus at around the same time. Irradiation with UV-B light represents one such stimulus.

We have previously reported that DTH to HSV-1 in a murine model can be suppressed by irradiation of the mice with 96 mJ/cm^2 ultraviolet-B (UV-B) light 3 days before sensitization with live virus (Howie, Norval & Maingay, 1986a). We have also demonstrated that epidermal antigen-presenting cells (APC) from mice similarly irradiated have a diminished ability to present HSV-1 antigens to HSV-1 T-helper cells *in vitro* (Howie, Norval & Maingay, 1986b). In order to investigate further the cellular nature of the UV-B-induced suppression of DTH to HSV-1 a series of cell transfer experiments was performed. The results of these experiments are presented here.

Abbreviations: APC, antigen-presenting cell; DTH, delayed-type hypersensitivity; HSV-1, herpes simplex virus type 1; PBS, Dulbecco's phosphate-buffered saline; T_{DH}, T effector cell for delayed-type hypersensitivity; TNCB, 1-chloro-2,4,6-trinitrobenzene; T_s, T-suppressor cell; UV-B, ultraviolet-B light.

Correspondence: Dr S. E. M. Howie, Dept. of Bacteriology, University Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

MATERIALS AND METHODS

Mice

C3Hf Bu/Kam female mice aged 10–12 weeks were used throughout. The mice were bred and maintained in the Department of Bacteriology animal house.

Virus

A clinical isolate of HSV-1, the DNA of which had been analysed using restriction endonucleases, was used throughout. The virus was cultured in Vero cells and assayed for plaque-forming units (p.f.u.) per ml as described previously (Howie, Norval & Maingay, 1986c).

TNCB

1-chloro-2,4,6-trinitrobenzene, picryl chloride containing 20% H_2O was purchased from BDH Ltd, Poole, Dorset. A 2% solution (w/v) was made up in acetone immediately before use.

Antibodies

Rat monoclonal anti-mouse Ly-1 (TIB 104) and anti-mouse Ly-2 (TIB 105) antibodies were purchased from the American Type Culture Collection. Rat anti-mouse Thy 1.2 (30H12) was the kind gift of Dr H. S. Micklem, Department of Zoology, Edinburgh University. Rabbit anti-rat IgG was purchased from Sigma U.K. Ltd, Poole, Dorset.

DTH assay

This has been described elsewhere (Howie *et al*, 1986a). In brief, mice were sensitized by subcutaneous injection in the right flank

with 5×10^6 p.f.u. HSV-1 grown in Vero cells. Control mice were sensitized with an equivalent number (10^4) of sonicated uninfected Vero cells. Mice were tested for DTH to HSV-1 between 8 days and 14 days after sensitization. Before challenge, mice were tail-marked with indelible ink and their ear thicknesses were measured. Challenge consisted of the equivalent of 10^6 p.f.u. of HSV-1, which had been inactivated by UV-B irradiation for 1 hr. The challenge was administered by injecting 10 μl UV-inactivated virus into each ear pinna. Twenty-four hours later, ear thickness was again measured and the DTH calculated as the average ear increase per mouse. In order to induce DTH to TNCB, mice were painted with 100 μl of a 2% solution of picryl chloride in acetone on shaved abdomens. Mice were challenged by painting 10 μl of a 2% solution of picryl chloride in acetone on both sides of the ear pinnae. Ear thickness was measured immediately before and 24 hr after challenge, as described for HSV-1 above.

Suppression of DTH

In order to induce suppression of DTH to HSV-1, mice were irradiated for 20 min under two Phillips TL20/12 bulbs, which gave a dose of 96 mJ/cm^2 on the shaved backs of the mice as described previously (Howie *et al.*, 1986a). Mice were irradiated in a high-sided perspex box in which they could not turn around. The ears of the mice were protected from UV-B irradiation by opaque tape, which did not allow any UV-light to penetrate. Suppression of DTH was determined by the formula:

$$\% \text{ suppression} = 100 - \frac{100 \times \text{net increase of UV mice}}{\text{net increase of control mice}}$$

as described by Noonan *et al.* (1984).

Cell transfers

Cells from spleens of HSV-1-sensitized mice and from mice irradiated with UV-B 3 days before sensitization with HSV-1 or TNCB were treated appropriately (see below) and injected intravenously into recipient mice 24 hr before ear challenge.

Cell separations

Cells were enriched for Thy-1+ve cells by passage over nylon-wool columns (Julius, Simpson & Herzenberg, 1973). This gav a population of cells >95% Thy-1+ve by immunofluorescenc Cells were depleted of Thy-1+ve cells by treatment with ant Thy 1.2 serum and guinea-pig complement. This gave population of cells <10% Thy-1+ve by immunofluorescenc For depletion of Ly-1 and Ly-2 bearing cells, nylon-wool passe spleen cells were treated with the appropriate monoclon antibodies. The rat monoclonal antibodies TIB 104 and TIB 10 were not cytotoxic. Spleen cells were incubated with thes antibodies for 1 hr at room temperature. They were then washe twice with PBS and incubated for 30 min at room temperatur with a rabbit anti-rat immunoglobulin serum (which had bee absorbed previously with mouse spleen cells to remove anti mouse activity), washed twice with PBS and incubated for 1 h at 37° in guinea-pig complement, which had been previousl absorbed with mouse spleen cells. Control cells for transfer wer treated as above with anti-rat Ig and complement only.

The percentage viable cells left after killing with the variou antisera was determined by trypan blue dye exclusion; for th anti-Ly-1 antibody (TIB 104) plus the anti-Ly-2 antibody (TI 105) this was $48 \pm 4\%$, for the anti-Ly-1 antibody alone $37 \pm 3\%$ for the anti-Ly-2 antibody alone $24 \pm 2\%$, and for the contro (i.e. cells treated with anti-rat Ig and complement only) $8 \pm 1\%$ These values were averaged over all experiments performec ±standard error.

RESULTS

DTH to HSV-1 is transferable by an Ly-1+ve 2−ve T cell

We have previously reported that antigen-specific DTH to HSV-1 can be induced by subcutaneous immunization with live virus (Howie *et al.*, 1986a). In order to determine which cell type was responsible for the DTH response, spleen cells from mice sensitized 8 days previously were transferred into naive syngeneic recipients. Twenty-four hours later, the ears of the recipient mice were challenged with UV-inactivated virus and the 24-hr DTH response measured. Table 1 shows that the cell responsible for the DTH response is an Ly-1$^+$2$^-$ T cell.

Table 1. Ly phenotype of transferable T_{DH}

Sensitization (Day 0)	Nylon-wool passed spleen cells transferred (Day 8)	Exp. 1: Increase in ear thickness (units × 10^{-2} mm)	Exp. 1: % maximum response	Exp. 2: Increase in ear thickness (units × mm^{-2})	Exp. 2: % maximum response
10^4	Nil	$3{\cdot}00 \pm 0{\cdot}37$ ($n=11$)	—	$2{\cdot}00 \pm 0{\cdot}2$ ($n=6$)	—
10^7 HSV-1	Nil	$14{\cdot}48 \pm 1{\cdot}02$ ($n=11$)¶	100	$10{\cdot}90 \pm 0{\cdot}89$ ($n=5$)¶	100
Nil	RIG+C only*	$10{\cdot}90 \pm 0{\cdot}69$ ($n=5$)¶	67	$9{\cdot}00 \pm 1{\cdot}25$ ($n=5$)¶	76
Nil	Ly1†	$3{\cdot}37 \pm 0{\cdot}39$ ($n=7$)	6	$2{\cdot}15 \pm 0{\cdot}33$ ($n=5$)	4
Nil	Ly2‡	$11{\cdot}17 \pm 1{\cdot}25$ ($n=6$)	71	$8{\cdot}25 \pm 0{\cdot}25$ ($n=5$)¶	70
Nil	Ly1+Ly2§	$3{\cdot}89 \pm 0{\cdot}89$ ($n=7$)	11	ND	ND

n = number of mice per group.

* Spleen cells from mice sensitized with HSV-1 8 days previously, passed over nylon wool and treated with anti-rat immunoglobulin and guinea-pig complement alone (3×10^6 cells transferred per mouse).

† Spleen cells as above but also treated with TIB 104 (3×10^6 cells transferred per mouse).

‡ Spleen cells as above but also treated with TIB 105 (3×10^6 cells transferred per mouse).

§ Spleen cells as above but also treated with TIB 104 and TIB 105 (3×10^6 cells transferred per mouse).

¶ Significantly different from control value ($P < 0{\cdot}001$ by Student's t-test).

Table 2. UV-B-induced suppression of DTH to HSV-1 is transferable by spleen cells

UV-B irradiation (Day −3)	Sensitization (Day 0)	No. spleen cells transferred i.v. (Day 8)	Increase in ear thickness (units × 10^{-2} mm)	% suppression
Nil	10^4 Vero	Nil	3·0 ± 0·35 ($n=10$)	—
Nil	10^7 HSV-1	Nil	11·25 ± 0·56 ($n=6$)†	—
96 mJ/cm²*	10^7 HSV-1	Nil	4·43 ± 0·53 ($n=4$)‡	83
Nil	10^7 HSV-1	3×10^7	5·8 ± 0·64 ($n=5$)‡	66
Nil	10^7 HSV-1	10^7	6·13 ± 1·45 ($n=5$)‡	62
Nil	10^7 HSV-1	3×10^6	10·70 ± 0·95 ($n=5$)	7
Nil	10^7 HSV-1	10^6	13·0 ± 0·87 ($n=5$)	—

All mice were challenged on Day 9 with 10^6 UV-inactivated p.f.u. HSV-1 in both ear pinnae; n = number of mice per group.

* This group of mice provided the spleen cells used for transfer.

† Significantly different from control value ($P<0·001$ by Student's t-test).

‡ Significantly different from positive response ($P<0·01$ by Student's t-test).

Spleen cells transfer the UV-B-induced suppression of efferent DTH to HSV-1

We have previously reported that DTH to HSV-1 can be specifically suppressed by irradiating dorsally shaved mice with 96 mJ/cm ² UV-B light 3 days before sensitization with HSV-1. This was a systemic effect that was not restricted to the irradiated site since the ears of the mice are protected by tape that allowed no penetration of UV-B (Howie *et al.*, 1986a). In order to determine which cells were responsible for the *in vivo* suppression of DTH, spleen cells from UV-B-suppressed mice were transferred to syngeneic recipients that had been sensitized to live virus 8 days previously. Twenty-four hours later, the mice were challenged in the ears with inactivated virus and the degree of suppression of DTH determined. Table 2 shows that spleen cells transfer the suppression of DTH in a titratable fashion.

The transferable UV-B-induced suppressor cell is a T cell

In order to determine which cell type was responsible for the transferable suppression, spleen cells from UV-B-suppressed animals were separated into T-cell enriched (nylon-wool passed) and T-cell depleted (anti-Thy-1- and complement-treated) populations. The separated cells were then injected intravenously into syngeneic recipients that had been sensitized with live virus 8 days previously. Twenty-four hours later, the ears were challenged with inactivated virus. Table 3 demonstrates that the suppressor cell is a T lymphocyte.

Ly-1⁺2⁻ and Ly-1⁻2⁺ T cells are responsible for the transfer of UV-R-induced suppression of DTH to HSV-1

Nylon-wool passed spleen cells from UV-B-suppressed mice

Table 3. The transferable UV-B-induced suppressor of DTH to HSV-1 is a T lymphocyte

UV-B irradiation (Day −3)	Sensitization (Day 0)	Spleen cells transferred (Day 8)	Increase in ear thickness (units × 10^{-2} mm)	% suppression
Nil	10^4 Vero	Nil	2·96 ± 0·58 ($n=10$)	—
Nil	10^7 HSV-1	Nil	11·43 ± 0·78 ($n=6$)§	—
96 mJ/cm²	10^7 HSV-1	Nil	3·65 ± 0·64 ($n=5$)¶	91
Nil	10^7 HSV-1	3×10^7 whole spleen*	4·97 ± 0·73 ($n=9$)¶	75
Nil	10^7 HSV-1	10^7 nylon wool spleen†	4·16 ± 0·94 ($n=6$)§	80
Nil	10^7 HSV-1	3×10^7 Thy-1 + C spleen‡	9·00 ± 0·39 ($n=8$)	28

n = number of mice per group.

* Spleen cells from mice irradiated with 96 mJ/cm² UV-B 8 on Day −3 and injected with 10^7 p.f.u. HSV-1.

† Spleen cells as above but passed over nylon wool (> 95% Thy-1 +ve by immunofluorescence).

‡ Spleen cells as above but treated with anti-Thy-1 monoclonal antibody and complement (< 10% Thy-1 +ve by immunofluorescence).

§ Significantly different from control value ($P<0·001$ by Student's t-test).

¶ Significantly different from positive response ($P<0·01$ by Student's t-test).

Table 4. Ly phenotype of the transferable UV-B-induced T_S for DTH to HSV-1

UV-B irradiation (Day −3)	Sensitization (Day 0)	Nylon-wool passed spleen cells transferred (Day 8)	Exp. 1* Increase in ear thickness (units × 10^{-2} mm)	Exp. 1* % suppression	Exp. 2† Increase in ear thickness (units × 10^{-2} mm)	Exp. 2† % suppression
Nil	10^4 Vero	Nil	3·00 ± 0·37 (n = 11)	—	1·5 ± 0·5 (n = 8)	—
Nil	10^7 HSV-1	Nil	14·48 ± 1·02 (n = 11)††	0	11·5 ± 1·3 (n = 6)††	0
96 mJ/cm²	10^7 HSV-1	Nil	5·38 ± 1·31 (n = 4)§§	78	4·0 ± 0·9 (n = 4)§§	74
Nil	10^7 HSV-1	RIG + C only‡	6·92 ± 0·56 (n = 6)‡‡	66	2·8 ± 0·8 (n = 5)‡‡	84
Nil	10^7 HSV-1	Ly1 treated§	8·63 ± 0·48 (n = 6)‡‡	51	4·0 ± 0·8 (n = 5)‡‡	74
Nil	10^7 HSV-1	Ly2 treated¶	6·89 ± 0·94 (n = 7)‡‡	66	4·7 ± 1·1 (n = 5)§§	66
Nil	10^7 HSV-1	Ly1 + Ly2 treated**	14·25 ± 0·88 (n = 7)	9	9·0 ± 1·5 (n = 5)	30

n = number of mice per group.

* No. of cells transferred in Exp. 1 = 3×10^6 per mouse.

† No. of cells transferred in Exp. 2 = 2×10^7 per mouse.

‡ Spleen cells from mice exposed to 96 mJ/cm² UV-B 11 days previously and 10^7 p.f.u. HSV-1 8 days previously, nylon-wool passed and treated with rat immunoglobulin and complement alone.

§ Spleen cells as in above but also treated with TIB 104.

¶ Spleen cells as in above but also treated with TIB 105.

** Spleen cells as in above but also treated with TIB 104 and TIB 105.

†† Significantly different from control value ($P < 0{\cdot}001$ by Student's t-test).

‡‡ Significantly different from positive response ($P < 0{\cdot}001$ by Student's t-test).

§§ Significantly different from positive response ($P < 0{\cdot}01$ by Student's t-test).

Table 5. Specificity of the UV-B-induced T_S cells

UV-B irradiation (Day −3)	Sensitization (Day 0)	Nylon-wool passed spleen cells transferred (Day 8)	Challenge* (Day 9)	Increase in ear thickness (units × 10^{-2} mm)	% suppression
Nil	Nil	Nil	HSV-1	2·25 ± 0·70 (n = 7)	—
Nil	Nil	Nil	TNCB	5·29 ± 0·78 (n = 7)	—
Nil	10^7 HSV-1	Nil	HSV-1	14·53 ± 0·69 (n = 9)	0
Nil	2% TNCB	Nil	TNCB	25·15 ± 1·06 (n = 5)	0
96 mJ/cm²	10^7 HSV-1	Nil	HSV-1	8·88 ± 0·72 (n = 4)	48
96 mJ/cm²	2% TNCB	Nil	TNCB	16·40 ± 1·04 (n = 5)	47
Nil	10^7 HSV-1	3×10^7, UV-B-HSV-1†	HSV-1	5·81 ± 0·47 (n = 4)	71
Nil	10^7 HSV-1	3×10^7, UV-B-TNCB‡	HSV-1	16·30 ± 1·20 (n = 5)	3
Nil	2% TNCB	3×10^7, UV-B-HSV-1	TNCB	25·30 ± 1·24 (n = 5)	6
Nil	2% TNCB	3×10^7, UV-B-TNCB	TNCB	16·55 ± 0·94 (n = 5)	46
Nil	10^7 HSV-1	3×10^6, UV-B-HSV-1, Anti-RIG + C§	HSV-1	7·15 ± 1·00 (n = 5)	60
Nil	10^7 HSV-1	3×10^6, UV-B-HSV-1, Ly1¶	HSV-1	8·00 ± 1·19 (n = 5)	51
Nil	10^7 HSV-1	3×10^6, UV-B-HSV-1, Ly2**	HSV-1	7·20 ± 1·00 (n = 5)	60
Nil	10^7 HSV-1	3×10^6, UV-B- HSV-1, Ly1††, Ly2	HSV-1	12·35 ± 0·73 (n = 5)	14
Nil	2% TNCB	3×10^6, UV-B-HSV-1, Ly1	TNCB	25·75 ± 1·08 (n = 5)	5
Nil	2% TNCB	3×10^6, UV-B-HSV-1, Ly2	TNCB	24·30 ± 1·32 (n = 5)	9

n = number of mice per group.

Mice were challenged either by injecting 10^6 p.f.u. UV-B-inactivated HSV-1 in both ear pinnae, or by painting both ear pinnae with 15 μl 2% TNCB in acetone.

† Nylon-wool passed spleen cells from mice treated with 96 mJ/cm² UV-B on Day −3 and HSV-1 s.c. on Day 0.

‡ Nylon-wool passed spleen cells from mice treated with 96 mJ/cm² UV-B on Day −3 and 100 μl 2% TNCB in acetone on shaved abdomen on Day 0.

§ Cells as above but treated with rabbit anti-rat Ig and complement only.

¶ Cells as above but treated with TIB 104.

** Cells as above but treated with TIB 105.

†† Cells as above but treated with TIB 104 and TIB 105.

/ere treated with (a) anti-Ly-1 serum, (b) anti-Ly-2 serum, (c) nti-Ly-1 and anti-Ly-2 serum, or (d) anti-rat Ig and comple-nent only, as described in the Materials and Methods. The reated cells were then transferred into syngeneic recipients that ıad been sensitized with live virus 8 days previously. After 24 hr he ears were challenged with inactivated virus and the 24-hr)TH response determined. Table 4 shows that both Ly-1$^+$2$^-$ nd Ly-1$^-$2$^+$ T suppressor cells are involved. Suppression was ınly abrogated by treating simultaneously with anti-Ly-1 and .nti-Ly-2 serum.

ipecificity of the transferable UV-B-induced T-suppressor cells of -)TH to HSV-1

n order to determine the specificity of the transferable T-uppressor cells, cross-over experiments were performed using nice sensitized to either HSV-1 or TNCB. Spleens from animals hat had been UV-B-irradiated 3 days before sensitization with ither HSV-1 or TNCB were removed 8 days after sensitization. The spleen cells were passed over nylon-wool columns and reated with antibodies to Ly-1 or Ly-2 antigens. Table 5 shows hat all the HSV-1 T-suppressor cell populations examined were pecific, in as much as they did not suppress DTH to TNCB. The ine specificity of the T cells in terms of their reactivity with other iral antigens is being examined.

DISCUSSION

)elayed-type hypersensitivity to HSV-1 has been extensively tudied in murine model systems (Nash, Field & Quartey-apafio, 1980; Schrier, Pizer & Moorhead, 1983a; reviewed in Nash, 1985). The DTH has been shown to be specific and under he control of the I region of the H-2 complex (Nash, Phelan & Wildy, 1981b). The DTH has been clearly demonstrated to be mportant in viral clearance (Nash *et al.*, 1981b). T-cell uppression of DTH to HSV-1 has also been extensively studied Nash, Gell & Wildey, 1981a; Shrier, Pizer & Moorhead, 1983b; eviewed in Nash, 1985).

The DTH response may be suppressed at either the afferent or efferent level, or at both levels. T-suppressor cells are esponsible for the suppression of both afferent and efferent)TH. The suppression of DTH to HSV-1 is specific in all cases. Shrier *et al.* (1983b) demonstrated that the intravenous inocula-ion of a temperature-sensitive mutant of HSV-1 would induce ransferable T-suppressor cells capable of suppressing both *fferent* and *efferent* DTH to HSV-1, whereas the injection of irally infected syngeneic spleen cells induced transferable T, vhich would only suppress *efferent* DTH. Using a non-athogenic strain of HSV-1 injected intravenously to induce uppression of DTH to HSV-1, Nash & Gell (1983) demon-trated that two phenotypically distinct T, cells were involved in uppression for *afferent* DTH to HSV-1, one being Ly-1$^+$2$^-$ and ne other Ly-1$^-$2$^+$.

Ultraviolet-B light is an external environmental stimulus nat has been implicated in triggering HSV-1 recrudescence Wheeler, 1975). UV-B is also a stimulus that is well known to nduce systemic T-suppressor cells of DTH specific for antigens een by the epidermis at a critical time after irradiation (Noonan *et al.*, 1984; Morrison, Bucana & Kripke, 1984). We have previously reported (Howie *et al.*, 1986a) that irradiation of mice with 96 mJ/cm^2 UV-B 3 days before subcutaneous injection with live pathogenic HSV-1 abrogates the DTH response normally generated. The present report demonstrates that this suppression is mediated by transferable T suppressor cells. Two phenotypically distinct T, subsets, one Ly-1$^+$2$^-$ and one Ly-1$^-$2$^+$, are responsible for the efferent suppression of DTH to HSV-1. Both T, subsets are 'specific', i.e. they do not suppress DTH to the skin-sensitizing agent, picryl chloride; our experiments to date do not, however, distinguish between a specific effector cell reaction or a specific induction of a non-specific event. We are examining this in greater detail.

The ability of a low (suberythemal) dose of UV-B irradiation to induce T, to epidermal antigens during a period of 2–10 days after irradiation (Howie *et al.*, 1986a) may be of crucial importance in determining the outcome of infection by epidermal pathogens during this time. In particular, when a persistent pathogen such as HSV-1 is involved, this may lead to a 'deviant' immunological balance being established between the host and the virus. Such an imbalance may be a factor in determining whether a primary infection becomes latent or in tipping the host–virus balance towards recrudescence of an already latent infection. We have also previously reported (Howie *et al.*, 1986b) that irradiation of the epidermis with 96 mJ/cm^2 UV-B light induces an alteration in the ability of epidermal cells to induce systemic T-suppressor cells of DTH specific for antigens seen by the epidermis at a critical time after irradiation (Noonan *et al.*, 1984; Morrison *et al.*, 1984). We have previously reported (Howie *et al.*, 1986a) that irradiation of mice with 96 mJ/cm^2 UV-B 3 days before subcutaneous injection with live pathogenic HSV-1 abrogates the DTH response normally generated. The present report demonstrates that this suppression is mediated by transferable T-suppressor cells. Two phenotypically distinct T, subsets, one Ly-1$^+$2$^-$ and one Ly-1$^-$2$^+$, are responsible for the efferent suppression of DTH to HSV-1. Both T, subsets are 'specific', i.e. they do not suppress DTH to the skin-sensitizing agent, picryl chloride; our experiments to date do not, however, distinguish between a specific effector cell reaction or a specific induction of a non-specific event. We are examining this in greater detail.

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The effect of UV-irradiation on viral infections of the skin

M Norval,* S Howie, J Ross & J Maingay

The effect on the skin of exposure to relatively small doses of ultraviolet light has long been thought beneficial and socially desirable, leading to a 'healthy tan'. However it is less well known that similar (if not smaller) doses of UV-B radiation can have transient but profound depressive effects on the immune response to antigens or pathogens encountered by the skin. The result of this on the immunopathology of both primary and recurrent episodes of persistent viral infections of the epidermis is discussed.

Skin as an immunologically competent organ

In recent years, the skin has been recognized as playing an important role in the recognition of, and response to, foreign antigens.[1,2] This immune surveillance is of crucial importance as, throughout life, the epidermis is constantly exposed to various insults including mechanical injury, chemicals such as irritants and contact sensitizing agents, physical agents like ultraviolet light (UV) as well as microbial pathogens.

The complexities of the interactions between the epidermal cells, the dermis, local lymph nodes and the peripheral immune system are beginning to be understood. Pivotal in this are the Langerhans cells (LCs) of the epidermis, first recognized by Paul Langerhans in 1868. These cells are known to act as the antigen presenting cells (APCs) of the immune system. They process foreign antigen and present it to T-lymphocytes in association with Class II antigens of the Major Histocompatibility Complex (MHC). LCs are dendritic in nature, bone-marrow derived and have the ability to move from the epidermis into the afferent lymphatics and so to the draining lymph nodes where they may act as dendritic APCs.[3] Epidermal keratinocytes produce an interleukin-1 like factor called epidermal-cell-derived thymocyte-activating factor (ETAF), which activates T-lymphocytes in association with antigen. In addition, they have been shown to express Class II MHC antigens after a variety of immune stimuli and exposure to γ-interferon in both mouse and man,[4,5] and may also process antigen. Recently interleukin-1 has also been shown to be synthesized by LCs.[6]

There is a population of T-lymphocytes present in the epidermis. These cells may be resident in that site or be attracted from the lymphatics or blood vessels of the skin by epidermal APCs. T-lymphocytes could recognize antigen presented by LCs within the epidermis and become effector cells locally. Alternatively, they could be activated in the lymph nodes by APCs (which have migrated there from the epidermis) and proliferate before moving to the skin to effect the immune response. In delayed type hypersensitivity (DTH) reactions both these events are thought to occur at different times during the development of the response.[7] Finally, a population of Thy-1 positive indeterminate cells, derived from the bone marrow, has been described in murine epidermis, which is postulated to consist of APCs for the induction of immunosuppression.[2] Figure 1 illustrates the structure of the epidermis.

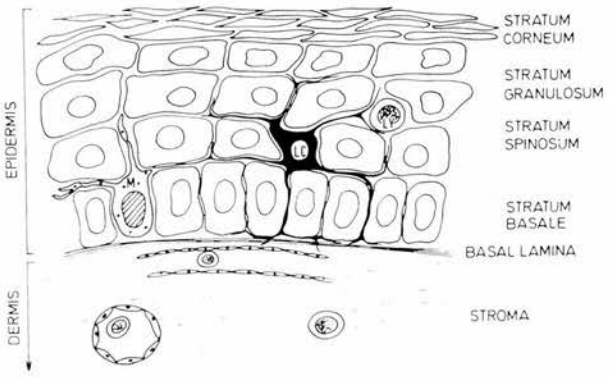

Figure 1. *Section of epidermis showing epidermal cells becoming more keratinized as they reach the surface, a Langerhans cell (LC), a melanocyte (M) and a lymphocyte (Ly). In the dermis are blood vessels, stromal cells and cells of the lymphatic system.*

Effect of UV radiation on immune function

Many systems have been described which demonstrate profound modulations of the immune system by UV radiation. These effects are complex and are made even more so by variations in the wavelength of UV light employed, the doses of radiation administered, the experimental animals studied and the parameters of the immune system measured. Mice have been used extensively as experimental models and the following results have all been obtained in murine systems. In essence, UV-irradiation results in the induction of immunosuppression with the generation of specific T suppressor (T_s) lymphocytes.

At the site of irradiation there is a local effect which is illustrated by a specific immunological tolerance to

*Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG.
Received 31 January, 1986.

heteroduplex analysis or Southern blot-hybridization.[32] These methods allow the study of plasmid evolution during an outbreak and demonstrate the presence and frequency of transposons carrying drug-resistance determinants from plasmid to plasmid.

Conclusions

Studies of the epidemic process in *S. sonnei* infections involve use of a series of microbiological methods, including phage typing, biotyping, colicin typing, and drug-resistance typing. They allow intraspecific subdivision of *S. sonnei* strains and provide support for epidemiological analysis in epidemic situations. Since phenotypic traits can experience change in the course of strain circulation, a detailed epidemiological investigation will require, in addition, examination of genetic relatedness between individual bacterial isolates. This is accomplished by plasmid pattern analysis, which is a valuable and reliable epidemiological laboratory method.

Acknowledgement

The author wishes to thank Dr A. Trifonova for critical reading of the manuscript.

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contact and photosensitizing agents.[8] It is known that UV-irradiation of certain wavelengths reduces the number of LCs in the epidermis in exposed areas, and changes the morphology (and perhaps the function) of those remaining;[9] in addition, ETAF production is markedly decreased. Recently, a new functional type of epidermal APC has been described,[10] which, unlike the LC, is UV-resistant and appears to induce, preferentially, T_s cells rather than T helper (T_h) lymphocytes. Such cells could induce T_s cells either locally or systemically, after migration from the epidermis to lymph nodes or the spleen.

In addition to the local effect of UV-irradiation, there is a systemic effect in which the immune response is suppressed at unexposed sites. The exact mechanism is not known but it is probable that several factors are involved. It takes time, several hours at least, for the effect to develop after the exposure. The distribution of LCs is altered; accumulations of APCs may occur in draining lymph nodes immediately following irradiation and other epidermal APCs may be destroyed because of DNA damage.[11] There is a local inflammatory response, the extent of which depends on the dose of UV-irradiation. This may deplete T-lymphocytes specific for DTH and suppress local graft-versus-host responses. However, the wavelength at which the LCs are altered (270–290 nm) can be separated from the wavelength at which the maximum suppression at an unexposed site occurs (320 nm),[12] so some other mediators must also be involved. The UV-spectrum is illustrated in Figure 2.

As already stated T_s cells are found following UV-irradiation which are antigen specific and which were first described as inducing systemic suppression in UV-induced tumour cells.[13] Presumably these are generated systemically. UV-irradiation has poor penetrating capacity and would only have effects on the upper cells of the epidermis. It is possible that DNA damage in these cells leads to the release of some chemical mediator(s) which could either interact locally with LCs, or circulate to lymphoid organs and there interact with APCs to induce the generation of T_s cells.[14] Urocanic acid as been suggested as a possible photoreceptor mediator.[15] This substance is located preferentially in the stratum corneum and absorbs UV at the same wavelength at which suppression occurs. UV-irradiation induces isomerization of urocanic acid from the *trans*- to the *cis*-form; at present no interaction of urocanic acid with the cells of the immune system has been demonstrated.

These various mechanisms are summarized in Table 1 and there is an excellent recent review by Kripke & Morison.[16]

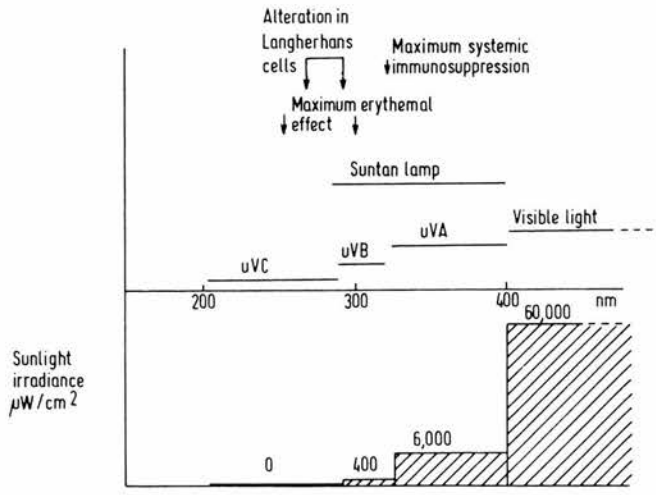

Figure 2. *Wavelength of radiation and points at which various effects occur.*

Table 1. *Immunosuppression caused by UV-irradiation.*

Local effect at exposed site	Systemic effect
UV-sensitive LCs ($I\text{-}A^+$): move, destroyed or function altered.	T_s cells induced by local effect.
UV-resistant LCs ($I\text{-}J^+$): induce T_s cells locally and/or systemically.	Redistribution of APCs.
ETAF production reduced. Prostaglandins PGE_2 and PGF_2 released from keratinocytes.	Chemical mediators released by DNA damage to epidermis or *cis*-isomer of urocanic acid interacts with APCs locally or systemically.

Herpes simplex virus UV-irradiation

So far, most studies on the modulation of the immune response by UV-irradiation have used a high dose (greater than 10000 J m^{-2}) which causes a marked erythema with extensive inflammatory changes in the skin. Systems include prevention of immunological rejection of skin cancers induced by UV-irradiation as already mentioned,[17] and contact hypersensitivity studies with various chemicals like dinitrofluorobenzene.[18] In contrast to these experiments, we wished to establish whether UV-irradiation altered the immune response to a viral infection of the skin and whether the dose of UV would have an effect if reduced to sub-erythemal levels. The virus chosen was herpes simplex (HSV).

HSV is a complex DNA-containing virus causing an epidermal infection of the oral or genital region. The infection is apparent clinically as 'cold sores' or may be subclinical. About 80% of adults have serological evidence of having been in contact with HSV, commonly as young children. After the primary infection, the virus is not eliminated from the body but persists in a latent form, probably integrated or as concatemerized viral DNA in the neurones of the ganglion serving the area of skin infected initially.[19] In about half of these cases, the virus reactivates at intervals, travels down the axon of the nerve and is found in the skin again. It may replicate there to form a recrudescent lesion.[20] The frequency of

these recrudescences varies enormously from one individual to another, from as often as once a month to once in a lifetime. Very often there is a recognizable triggering factor and one of the most common for oral HSV recrudescence is exposure to UV-light.

The importance of the immune system in controlling primary and latent HSV infections has been demonstrated under both experimental and natural conditions and has been reviewed extensively.[21,22] However, the relative roles of the various facets of the immune response, specific and non-specific, humoral and cell-mediated, remain unclear. It seems likely that epidermally encountered pathogens like HSV are processed and presented to specific T-lymphocytes on the surface of LCs, although whether HSV can productively infect and destroy LCs, thus altering antigen presentation in the skin, is not known.

We examined the effect of UV-irradiation on the generation of immunity to primary HSV in a murine model. Mice were irradiated with a suberythemal dose of UV-B (wavelength 270–350 nm, dose 960 J m^{-2}) on their shaved dorsal skin 3 d before being infected subcutaneously with HSV. They were challenged 8 d later with inactivated HSV and were found to have 60–90% suppressed DTH response compared to non-irradiated control mice.[23] DTH is particularly important in viral clearance from a local site. Irradiation on the same day as infection had no effect on DTH, showing that the suppression is not immediate. However, the infection had to take place within a limited time (14 d) after UV-irradiation, otherwise no suppression was generated. Once the suppression was induced, it persisted for as long as tested (more than 30 d).

To test the nature of the suppression, spleen cells from UV-irradiated mice were transferred to mice which had been infected with virus 8 d before. Suppression of DTH resulted and it was specific for HSV. The suppression was shown to be due to T_s cells in the transferred spleen cell population and two phenotypically distinct subsets were involved, distinguishable on the basis of their cell surface antigen expression (S. Howie, M. Norval, J. Maingay and J. Ross, unpublished results).

To examine antigen presentation of HSV by epidermal cells, an *in vitro* assay was set up. It was found that UV-irradiation of mice before the epidermal cells were collected decreased antigen presentation by at least 60%. Interestingly, this reduction was not only a local effect on the epidermal cells at the exposed site but was found in epidermal cells from unexposed sites also. However, the APC capacity of peritoneal cells was unaffected by UV-irradiation.[24] We do not know what this defect in antigen presentation is: it may be a functional reduction, a reduction in numbers of APCs in the epidermis or an alteration in trafficking properties.

Preliminary experiments on the photo-isomer of urocanic acid have proved unexpectedly exciting. Injection of this compound subcutaneously, followed by sensitization with HSV, led to suppression of DTH response on subsequent challenge with HSV. This suppression was of the same order as that induced by UV-irradiation and experiments are in progress to ascertain if *cis*-urocanic acid affects the epidermal function of APCs, numbers, trafficking out of the epidermis or whether it might affect the generation of T_s cells systemically.

Conclusions and speculations

Given the importance of the immune system in controlling HSV infections, any environmental stimulus which transiently depresses the immune response may have profound effects on the nature of the host-virus balance, depending on the timing of the two events. Thus UV-irradiation in a critical period prior to contact with HSV may determine the severity and extent of primary infection, which infections become latent and which recrudesce and with what frequency and severity. In our experiments, low-dose irradiation was used. It is probable that continuous exposure to high doses of UV, as in tropical countries, may lead to a reduction in immunosuppressive effect, perhaps by changes in the permeability of the epidermis or by the synthesis of a greater amount of melanin.

There are other epidermal viral infections, in addition to HSV, which are of interest in the context of immunosuppression. Varicella zoster causes chicken pox; the virus becomes latent and may recrudesce to produce shingles many years later, often when the immune system is thought to be depressed. Skin warts are the result of infection by several types of papilloma viruses. Warts persist for many weeks or months, then frequently regress spontaneously. Cell-mediated immunity is thought to be important in their control and cure, and certainly immunosuppressed patients are recognized as having a high incidence of papilloma infection, often of a florid nature.[25] There is growing evidence of latent infection of epidermal cells after the wart has disappeared and the histopathological appearance is normal. Various pox viruses, such as molluscum contagiosum and orf, cause epidermal infections and the host response to these, often persistent in nature, is not well understood. Other viruses have epidermal phases which can be very transient, as in rubella, or longer lasting, as in measles. For all of these infections, the effect of UV-irradiation around the time of the initial contact with the virus upon severity of symptoms, persistence of virus, latency or any second phase of infection is not known.

Acknowledgement

This work was supported by the Medical Research Council of Great Britain of which S.H. is a Senior Research Fellow.

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IUMS News

Microbe '86

XIV International Congress of Microbiology, 7–13 September 1986, Manchester, UK.

This important Congress will be held under the auspices of the IUMS and under the joint sponsorship of The Royal Society of London, The Society for General Microbiology, The Society for Applied Bacteriology, The Society for Chemical Industry and The British Mycological Society.

The scientific programme incorporates the Bacteriology Division Meeting, which offers 32 symposia highlighting topics including systematics, medicine, veterinary science, genetics, molecular biology, ecology and industrial/applied microbiology (*IUMS News*, May 1986). The Mycology Division Meeting offers 10 symposia covering genetics, mycotoxins, membranes and mycorrhiza (*IUMS News*, April 1986). Details of the Social Programme (organized by Dr TA Roberts) and the Virology Programme (Dr BWJ Mahy) will appear in the August 1986 issue of *Microbiological Sciences*.

Further information can be obtained from the Secretary to the Organizing Committee: Dr JA Cole, Department of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK.

The 2nd Circular for the Congress is available from Mr AF Yates, Trading Services, UMIST, PO Box 88, Sackville Street, Manchester M60 1QD, UK.

Xth International Specialized Symposium on Yeast

The Xth International Specialized Symposium on Yeast 'Genetics and Molecular Biology' co-sponsored by IUMS was organized by the Institute of Molecular Biology, Bulgarian Academy of Sciences for the International Commission for Yeasts at Varna, Bulgaria on 4th–9th November, 1985.

This highly successful Symposium was attended by 148 participants from almost all European countries, Canada, the USA and Japan.

IUMS financial support was used to assist 17 participants, 3 from USA, 3 from USSR, 2 from The Netherlands, 2 from Canada and 1 each from Norway, France, Switzerland, Denmark, Japan and the UK.